REMARKS

Claims 1, 3-5, 9-13, 15, 17-21, 23, 25-27, 29-31, 33, and 35-39 were pending in the application. Claims 1, 17-18, 23, and 35-36 have been amended. Accordingly, upon entry of the instant response, claims 1, 3-5, 9-13, 15, 17-21, 23, 25-27, 29-31, 33, and 35-39 will remain pending in the application.

Claims 1, 17-18, 23, and 35-36 have been amended to specify "an antigen binding fragment," as suggested by the Examiner. Support for this amendment can be found throughout the specification and the claims as originally filed. Specifically, support is present, at least, for example, at page 11, lines 7-29.

No new matter has been added. The foregoing claim amendments should in no way be construed as an acquiescence to any of the Examiner's rejections and were made solely in the interest of expediting prosecution of the application. Applicants reserve the right to pursue claims covering any subject matter canceled herein in this or a separate application(s).

Acknowledgment of the Examiner's Withdrawal of Certain Rejections and Objections

Applicants gratefully acknowledge the Examiner's withdrawal of (a) the previous rejection of claims 1, 4, 9-10, 12-13, 15, 17-21, 23, 26, 29-31, 33 and 35-39 under 35 U.S.C. § 103(a) as being unpatentable over Foster *et al.* (U.S. Patent 5,217,954) in view of Hagiwara *et al.* (U.S. Patent No. 6,165,467); and (b) the previous rejection of claims 1, 3-4, 6, 9-15, 21, 23, 25-26, 28-33 and 39 under 35 U.S.C. § 103(a) as being unpatentable over Kerwin *et al.* (U.S. Patent 5,929,031) in view of Hagiwara *et al.* (U.S. Patent No. 6,165,467). Further, as the Examiner has not reiterated the objection to claims 1, 3, 4, 9-13, 15, 17-21, 23 and 28 for minor informalities (*i.e.*, as reading on non-elected species), Applicants respectfully assume that this objection has been withdrawn.

Information Disclosure Statements

Applicants gratefully acknowledge the Examiner's indication that the Information Disclosure Statements and accompanying PTO/SB/08 Forms submitted on October 19, 2006, January 18, 2007, May 16, 2007 and December 5, 2007 have been considered.

Rejection of Claims Under 35 U.S.C. § 112, First Paragraph

Claims 1, 3-5, 9-13, 15, 17-21, 23, 25-27, 29-31, and 35-39 are rejected under 35 U.S.C. § 112, first paragraph, as not being enabled. The Examiner acknowledges that the instant specification is enabling for an antibody formulated with DTPA and DEF. However, the Examiner alleges that the specification is not enabling for a composition comprising a fragment of an antibody formulated with DTPA and DEF because such fragments "encompass, for example, a single amino acid residue of an antibody."

To expedite prosecution, Applicants have amended claims 1, 17, 18, 23, 35 and 36 to specify "an antigen binding fragment thereof," as suggested by the Examiner. Accordingly, the Examiner's assertion that "fragments" are not enabled is now moot.

The Examiner further asserts that the specification is not enabling for a pharmaceutical composition (*i.e.*, claims 21 and 39) comprising an antibody (or fragment thereof) formulated with DTPA and a high concentration of DEF.

Applicants respectfully traverse this rejection and submit that an appropriate concentration of DEF can be determined by one of ordinary skill in the art based on the knowledge available in the art at the time of filing and the teachings set forth in Applicants' specification. For example, as pointed out by the Examiner, a "composition comprising a high concentration of deferoxamine would not be used by one of skill in the art for pharmaceutical purposes" in view of the teachings set forth in U.S. Patent No.: 5,268,165" (hereinafter the '165 patent), which discloses that DEF can "potentially caus[e] hypotension when administered intravenously" and "[s]ide effects from fast intravenous injection of...[deferoxamine]...may lead to dramatic blood pressure drop." Accordingly, to avoid these known undesirable side effects, one of ordinary skill in the art would follow the art-recognized recommended concentrations of DEF, *e.g.*, for intravenous administration of proteins. In addition to U.S. Patent No.: 5,268,165, other publications evidencing that these guidelines were well-established prior to the filing of the present invention include, for example, the enclosed publications by Bergeron *et al.* (*Blood* (2002) Apr 15;99(8):3019-26; Appendix A), Kacew (*Drug Toxicity and Metabolism in Pediatrics* (1990) Chapter 16: 295-305; Appendix B) and Christensen (*Hum Exp Toxicol.* 2001 Jul;20(7):365-72; Appendix C). Specifically, these publications shown that it was known prior to the filing of the present application that intravenous infusion of deferoxamine at a dose of 15 mg/kg/h is safe and <u>not</u> associated with hypotension. Similarly, guidelines for the safe administration of DEF via others means, such as oral or intramuscular administration or

intramuscular, were also well-established prior to the filing of the present application. Accordingly, one of ordinary skill in the art could readily establish the appropriate dose of the claimed composition containing DEF based on recommended standards known in the art prior to Applicants' invention.

Additionally, one of ordinary skill in the art could have determined the appropriate concentrations of DEF for administration to a subject in light of the teachings provided in Applicants' specification. Specifically, at page 14 (lines 8-23) of the specification, Applicants teach that appropriate doses of the claimed compositions depends upon a number of factors (*e.g.*, age, body weight, general health, gender, and diet of the subject, time of administration, route of administration, *etc.*) within the knowledge of the ordinarily skilled physician, veterinarian, or researcher. Applicants further teach guidelines for dosage unit forms (see page 17, lines 7-15), suitable routes of administration (see page 14, line 24 through page 15, line 2), suitable formulations (page 15, line 3 through page 16, line 6).

Accordingly, in view of the teachings in Applicants' specification and knowledge available in the art at the time of filing, the presently claimed compositions were fully enabled under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1, 3-5, 9-10, 12-13, 15, 17-21, 23, 25-27, 29-31, 33 and 35-39 Under 35 U.S.C. § 103

Claims 1, 3-5, 9-10, 12-13, 15, 17-21, 23, 25-27, 29-31, 33 and 35-39 are rejected as being unpatentable over Foster *et al.* (U.S. Patent 5,217,954) in view of Hagiwara *et al.* (U.S. Patent No. 6,165,467), Packer *et al.* (*Methods Enzymol*, 186: 41-42 (1990)) and Akers (*J. Par. Sci. Tech.* 36:222-228 (1982)). The Examiner relies on Foster *et al.* for teaching the use of a pharmaceutical formulation comprising a protein, bFGF, a stabilizing chelator, such as DTPA or EGTA to protect bFGF from oxidation. The Examiner further relies on Foster *et al.* for teaching an agent for tonicity, a preservative or other auxillaries, such as mannitol, glycerol, sodium chloride or Tris. The Examiner relies on Hagiwara *et al.* for teaching a stable human monoclonal antibody preparation and that human monoclonal antibodies have an undesirable property that they easily aggregate and precipitate in a solution state. The Examiner relies on Packer *et al.* for teaching that DEF suppresses iron-dependent generation of OH from H_2O_2. The Examiner relies on Akers for teaching that the use of a combination of antioxidants in the same formulation produces a synergistic effect. Although the Examiner acknowledges that Foster do

not teach a composition comprising an antibody formulated with DTPA and DEF, the Examiner concludes that it would have been obvious to one of skill in the art that "the use of DTPA and DEF would stabilize a composition comprising a human monoclonal antibody" in view of the cited references.

Applicants respectfully traverse this rejection. According to the obviousness standard articulated in *KSR Int'l Co. v. Teleflex Inc.* (127 S. Ct. 1727 (2007)) (hereinafter "*KSR*"), an invention composed of several elements is not proved obvious simply by demonstrating that each of its elements was independently known in the prior art. Specifically, the Court in *KSR* noted that *the combination must be evidenced from the prior art*, and a variety of factors (*e.g.*, interrelated teachings of multiple patents, demands in the marketplace, background possessed by one of ordinary skill in the art) must be considered "[t]o determine whether there was an apparent reason to combine the known elements in the way a patent claims (emphasis added)." Moreover, § 103 is a bar to patentability when a person of ordinary skill can implement a "predictable variation."

In accordance with this legal framework, one of ordinary skill in the art would not have been motivated to have combined the teachings of the cited references to arrive at the presently claimed compositions. Although Foster *et al.* and Packer *et al.* independently teach that DTPA and DEF have stabilizing properties, there was no teaching in the art prior to Applicants' filing that would have made it predictable that that these two agents, in particular, would be especially effective at preventing degradation of an antibody, when combined together. In fact, there was no teaching or suggestion in the prior art whatsoever that there would be a benefit to combining these two agents from amidst the many stabilizing agents known in the art. The mere fact that Akers generally teaches that combinations of antioxidants *can* produce a synergistic effect on the antioxygenic behavior of one or both antioxidant compounds would not have motivated one of ordinary skill in the art to arbitrarily try combining DEF with DTPA, when hundreds of other antioxidant compounds were known in the art. Further, since Foster *et al.* and Packer *et al.* had already discovered that DTPA and DEF in their own right can successfully protect a protein against degradation, one of ordinary skill in the art would have had no reason to combine these two specific agents.

Further, the claimed compositions are not merely a "predictable variation" arising from the combined teachings of the cited references. As taught in Applicants' specification, Applicants were the first to discover the unexpected and surprising synergistic effect that the

specific combination of DTPA and DEF, in particular, has on reducing/preventing protein oxidation (see, for example, page 2, lines 23-25; page 4, lines 10-13; page 27, line 7 through page 8, line 23; and page 36, lines 27-32 of the specification). In contrast to the suggestion in Akers that any combination of antioxidants would produce a synergistic effect, Applicants did not find this to be the case with other combinations of other agents, such as EGTA and DEF. While Applicants did note that the combination of EGTA and DEF conferred some level of protective effect (see, for example, page 2, lines 24-25, page 4, lines 6-10; page 14, lines 4-7; page 28, lines 26 through page 29, line 10 of the specification), the combination did not result in a significant effect. Therefore, contrary to the suggestion of Akers, the mere fact that two anti-oxidative agents are combined does not mean the resulting combination will produce a significant effect. As such, Applicants' discovery that the unique combination of EGTA and DEF yields a synergistic protective effect was not predictable in view of the teachings of the cited references and, as such, was not *prima facie* obvious in light of the standard established under *KSR*.

Based at least on the foregoing, the pending claims are patentable. Accordingly, Applicants respectfully request the Examiner to reconsider and withdraw the rejection.

Rejection of Claims 1, 3-5, 9-13, 12-13, 15, 17, 18, 21, 23, 25-27, 29-31, 35, 36, and 39 Under 35 U.S.C. § 103

Claims 1, 3-5, 9-13, 12-13, 15, 17, 18, 21, 23, 25-27, 29-31, 35, 36, and 39 are rejected as being unpatentable over Kerwin *et al.* (U.S. Patent 5,929,031) in view of Hagiwara *et al.* (U.S. Patent No. 6,165,467), Packer *et al.* (*Methods Enzymol*, 186: 41-42 (1990)) and Akers (*J. Par. Sci. Tech.* 36:222-228 (1982)). The Examiner relies on Kerwin *et al.* for teaching the preparation of a pharmaceutical composition which includes hemoglobin, a reducing agent, one or more chelators (such as DTPA or EGTA) and, optionally, one or more buffers (such as citrate of Tris) and salts (such as sodium chloride). The Examiner relies on Hagiwara *et al.*, Packer *et al.* and Akers for the reasons discussed above. Although the Examiner acknowledges that Kerwin *et al.* do not teach an antibody formulated with DTPA and DEF, the Examiner asserts that it would have been obvious to one of skill in the art that "the use of DTPA and DEF would stabilize a composition comprising a human monoclonal antibody" in view of the cited references.

Applicants respectfully traverse this rejection for the reasons discussed above with respect to the obviousness rejection over Foster *et al.* in view of the cited secondary references.

Specifically, in view of the standard set forth by the Court in *KSR* (summarized above), one of ordinary skill in the art would not have had reason to combine the teachings of the cited references to arrive at the presently claimed compositions. Although Kerwin *et al.* and Packer *et al.* independently teach that DTPA or EGTA and DEF have stabilizing properties, there was no teaching in the art prior to Applicants' filing that would have made it predictable that that these two agents, in particular, would be especially effective at preventing degradation of an antibody, when combined together. In fact, there was no teaching or suggestion in the prior art whatsoever that there would be a benefit to combining these two agents from amidst the many stabilizing agents known in the art. The mere fact that Akers generally teaches that combinations of antioxidants *can* produce a synergistic effect on the antioxygenic behavior of one or both antioxidant compounds would not have motivated one of ordinary skill in the art to arbitrarily try combining DEF with DTPA, when hundreds of other antioxidant compounds were known in the art. Further, since Kerwin *et al.* and Packer *et al.* had already discovered that DTPA and DEF in their own right can successfully protect a protein against degradation, one of ordinary skill in the art would have had no reason to combine these two specific agents.

Further, as discussed above, the claimed compositions are not merely a "predictable variation" arising from the combined teachings of the cited references. As taught in Applicants' specification, Applicants were the first to discover the unexpected and surprising synergistic effect that the specific combination of DTPA and DEF, in particular, has on reducing/preventing protein oxidation (see, for example, page 2, lines 23-25; page 4, lines 10-13; page 27, line 7 through page 8, line 23; and page 36, lines 27-32 of the specification). In contrast to the suggestion in Akers that any combination of antioxidants would produce a synergistic effect, Applicants did not find this to be the case with other combinations of other agents, such as EGTA and DEF. While Applicants did note that the combination of EGTA and DEF conferred some level of protective effect (see, for example, page 2, lines 24-25, page 4, lines 6-10; page 14, lines 4-7; page 28, lines 26 through page 29, line 10 of the specification), the combination did not result in a significant effect. Therefore, contrary to the suggestion of Akers, the mere fact that two anti-oxidative agents are combined does not mean the resulting combination will produce a significant effect. As such, Applicants' discovery that the unique combination of EGTA and DEF yields a synergistic protective effect was not predictable in view of the

teachings of the cited references and, as such, was not *prima facie* obvious in light of the standard established under *KSR*.

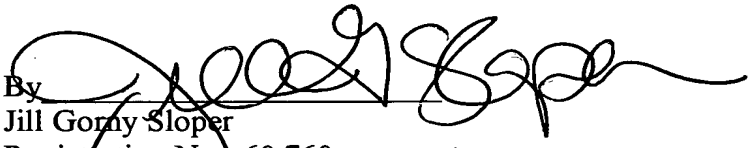
Based at least on the foregoing, the pending claims are patentable. Accordingly, Applicants respectfully request the Examiner to reconsider and withdraw the rejection.

CONCLUSION

In view of the foregoing, entry of the amendments and remarks herein, reconsideration and withdrawal of all rejections, and allowance of the instant application with all pending claims are respectfully solicited. If a telephone conversation with Applicants' attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call Applicants' attorney at (617) 227-7400.

Dated: April 15, 2008

Respectfully submitted,



By
Jill Gormy Sloper
Registration No.: 60,760
LAHIVE & COCKFIELD, LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 227-7400
(617) 742-4214 (Fax)
Attorney/Agent For Applicant

Appendix A

blood

2002 99: 3019-3026
doi:10.1182/blood.V99.8.3019

HBED ligand: preclinical studies of a potential alternative to deferoxamine for treatment of chronic iron overload and acute iron poisoning

Raymond J. Bergeron, Jan Wiegand and Gary M. Brittenham

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

HBED ligand: preclinical studies of a potential alternative to deferoxamine for treatment of chronic iron overload and acute iron poisoning

Raymond J. Bergeron, Jan Wiegand, and Gary M. Brittenham

We have continued the preclinical evaluation of the efficacy and safety of the hexadentate phenolic aminocarboxylate iron chelator *N, N'*-bis(2-hydroxybenzyl) ethylenediamine-*N, N'*-diacetic acid monosodium salt (NaHBED) for the treatment of both chronic transfusional iron overload and acute iron poisoning. We examined the effect of route of administration by giving equimolar amounts of NaHBED and deferoxamine (DFO) to *Cebus apella* monkeys as either a subcutaneous (SC) bolus or a 20-minute intravenous (IV) infusion. By both routes, NaHBED was consistently about twice as efficient as DFO in producing iron excretion. For both chelators at a dose of 150 μmol/kg, SC was more efficient than IV administration. The biochemical and histopathologic effects of NaHBED administration were assessed. No systemic toxicity was found after either IV administration once daily for 14 days to iron-loaded dogs or after SC administration every other day for 14 days to dogs without iron overload. Evidence of local irritation was found at some SC injection sites. When the NaHBED concentration was reduced to 15% or less in a volume comparable to a clinically useful one, no local irritation was found with SC administration in rats. Because treatment of acute iron poisoning may require rapid chelator infusion, we compared the effects of IV bolus administration of the compounds to normotensive rats. Administration of DFO produced a prompt, prolonged drop in blood pressure and acceleration of heart rate; NaHBED had little effect. NaHBED may provide an alternative to DFO for the treatment of both chronic transfusional iron overload and of acute iron poisoning. (Blood. 2002;99:3019-3026)

Introduction

Over the past 3 decades, deferoxamine B (DFO, Figure 1) has demonstrated its iron-chelating efficacy for the treatment of both chronic iron overload and acute iron poisoning.[1,2] For patients who require chronic transfusion for thalassemia, sickle cell disease, myelodysplasia, or other refractory anemias, DFO has been a generally safe and effective therapeutic agent that can control body iron; alleviate hepatic, cardiac, and endocrine dysfunction; improve growth and sexual maturation; and extend survival.[3-6] In the treatment of acute iron poisoning, DFO may be lifesaving. Enteral administration can bind unabsorbed iron in the gastrointestinal tract, and intravenous (IV) infusion can help clear iron from the systemic circulation.

Despite this admirable record, the limitations of DFO as an iron-chelating agent have also become evident. For patients with chronic iron overload, treatment with DFO is costly, inefficient, cumbersome, and unpleasant. The siderophore is still produced by large-scale fermentation of a strain of *Streptomyces pilosus*,[7] contributing to the expense of the drug. DFO is inefficient as an iron chelator; typically only 5% or less of the drug administered promotes iron excretion. Because gastrointestinal absorption is poor and circulatory elimination is rapid, effective therapy for chronic iron overload usually requires subcutaneous (SC) or IV administration by a portable infusion pump for 9 to 12 hours for 5 or 6 days each week.[8-10] Not surprisingly, most patients have difficulty in complying with such a demanding regimen. Moreover, a variety of toxic effects of treatment with DFO have become evident.[11-13] Allergy to DFO is rare but develops in some patients[14,15]; painful reactions at the site of SC infusion are nearly universal.[16,17] Other adverse reactions include visual and auditory neurotoxicity,[18] pulmonary toxicity,[19] bony changes,[20] growth failure,[21] and promotion of *Yersinia enterocolitica* infections.[22] Although improved management strategies have decreased the prevalence of neurotoxicity, bony changes, and growth failure, compliance with DFO remains an important problem.[1]

Although slow IV infusions of DFO (< 10 mg/kg per hour) have been well tolerated in patients with chronic iron overload,[23-25] rapid IV administration for the treatment of acute iron poisoning can produce severe hypotension.[26] In addition, IV infusions of DFO at doses higher than the recommended 15 mg/kg per hour for more than 24 hours for therapy of acute iron poisoning have resulted in several instances of acute respiratory distress syndrome (ARDS).[19,27] ARDS has also been reported in a child who had been treated with IV DFO according to the current guidelines.[28] Finally, the manufacturer recommends that the total daily parenteral dose of DFO should not exceed 6 g. Because 1 g DFO only binds about 90 mg elemental iron, and a single tablet for the treatment of iron deficiency in adults may contain as much as 65 mg elemental iron,

From the Department of Medicinal Chemistry, University of Florida, Gainesville, and the Departments of Pediatrics and Medicine, Columbia University College of Physicians and Surgeons, New York, NY.

Submitted February 13, 2001; accepted November 29, 2001.

Supported in part by research grants from the National Institutes of Health (DK49108, DK57209, and HL62882) and by Geltex, Waltham, MA.

R.J.B. and G.M.B. serve as consultants to Geltex Pharmaceuticals, which has licensed the patent for HBED from the University of Florida, Gainesville. G.M.B. also serves as consultant to Watson Pharmaceuticals, which has a sublicense for the development of HBED from Geltex Pharmaceuticals. J.W. has received honoraria from GelTex.

Reprints: Raymond J. Bergeron, Box 100485 JHMHC, Department of Medicinal Chemistry, University of Florida, Gainesville, FL 32610; e-mail: bergeron@mc.cop.ufl.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

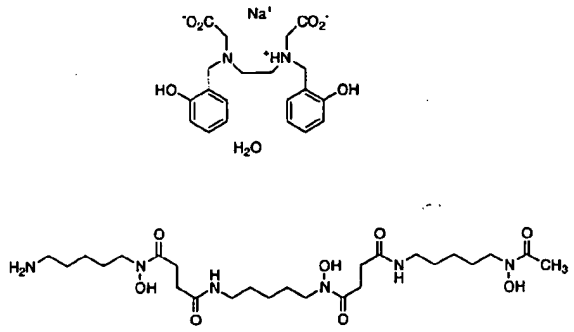


Figure 1. Structures of the iron chelators chosen for evaluation. *N, N'*-bis(2-hydroxybenzyl)ethylenediamine-*N, N'*-diacetic acid monosodium salt (NaHBED, top), and deferoxamine B (DFO, bottom).

6 g DFO given parenterally may not be adequate in patients with severe iron poisoning.

N, N'-Bis(2-hydroxybenzyl)ethylenediamine-*N, N'*-diacetic acid (HBED, Figure 1) is a synthetic hexadentate ligand that, like DFO, forms a 1:1 complex with iron with high affinity and selectivity.[29] We have previously reported studies of the efficacy and safety of parenteral administration of this synthetic iron chelator, predominantly as its monosodium salt (NaHBED). For the treatment of acute iron poisoning, NaHBED administered orally would bind and inactivate iron in the gastrointestinal tract and prevent its absorption. Administered IV, NaHBED would also bind and inactivate excess iron in the systemic circulation but without producing the hypotension that results from rapid IV administration of DFO. For treatment of chronic iron overload, we anticipate that administration of NaHBED would require a single SC or IV injection once every other day or, perhaps, once or twice weekly. This schedule of parenteral administration might be preferable to daily prolonged SC infusions of DFO or to ingestion of a large number of tablets on several occasions daily. Other efforts to develop substitutes for DFO for chronic iron-chelating therapy have concentrated on bidentate or tridentate ligands to be administered orally.[30,31] HBED, as a hexadentate chelator, would be free of the risk of exacerbating iron-related tissue damage that may be present with some of these partial ligands. Because HBED is a synthetic product, problems of local reactions to potential fermentation by-products not removed during purification would be absent. Finally, for patients allergic to DFO, HBED, a member of a different family of chelators, would be unlikely to provoke a similar response.

In our earlier studies, we found that SC administration of HBED to iron-loaded monkeys consistently induced iron clearance that was 2 to 3 times greater than that induced by an equimolar dose of DFO and have observed no adverse effects of chelator administration.[32,33] We now report further preclinical investigations of this compound, including (1) a comparison of the iron clearing efficiency of IV versus SC administration of NaHBED in primates, (2) the systemic toxicity of IV NaHBED in dogs, (3) the systemic toxicity of SC NaHBED in dogs, (4) an assessment of the local tolerability of SC NaHBED in rodents, and (5) the impact of IV NaHBED on the blood pressure and heart rate of normotensive rodents. Based on the data available thus far, NaHBED, administered IV as well as SC, continues to be a strong candidate for a much-needed alternative to DFO that may provide patients with chronic iron overload or acute iron poisoning with a safe, clinically effective form of iron-chelating therapy.

Materials and methods

Materials

Deferoxamine B in the form of the methanesulfonate salt, Desferal, was obtained from Novartis Pharma (Basel, Switzerland). The monosodium salt of HBED was obtained from either Strem Chemical (Newburyport, MA) or Prime Organics (Lowell, MA). Sprague-Dawley rats (Crl:CD(SD)BR-CD) were purchased from Charles River (Wilmington, MA). *Cebus apella* monkeys were obtained from World Wide Primates (Miami, FL). Male beagle dogs were purchased from Harlan Sprague-Dawley (Indianapolis, IN). All reagents and standard iron solutions were obtained from Aldrich Chemical (Milwaukee, WI). Atomic absorption measurements were made on a Perkin-Elmer model 5100 PC (Norwalk, CT). Ultrapure salts were obtained from Johnson Matthey Electronics (Royston, United Kingdom). All hematologic and biochemical studies[34] were performed by Antech Diagnostics (Tampa, FL). Histologic evaluation of necropsy tissues was performed by Florida Vet Path (Bushnell, FL).

Iron loading of *C apella* monkeys

The monkeys were overloaded with IV iron dextran as previously described to provide about 500 mg Fe/kg body weight[35]; the serum transferrin iron saturation rose to between 70% and 80%. We waited at least 20 half-lives, 60 days,[36] before using any of the animals in experiments evaluating iron-chelating agents.

Iron-balance studies in *C apella* monkeys

Seven days before the administration of the drug, the animals were placed in metabolic cages[37] and started on a low-iron liquid diet.[34] The monkeys were maintained on the low-iron liquid diet for the duration of the experiment. They were given food according to their body weight, and intake was very carefully monitored.

The total amount of iron intake was compared with the total amount of iron excreted: net iron balance = dietary iron intake − (urinary + fecal iron excretion). Animals in a negative iron balance are excreting more iron than they are absorbing.

Primate fecal and urine samples

Fecal and urine samples were collected at 24-hour intervals and processed as given in detail in earlier publications[32-34,38] before analysis by flame atomic absorption.

Drug preparation and administration to primates

Deferoxamine was dissolved in sterile water at a concentration of 50 or 100 mg/mL and given to the monkeys SC at a volume of 1 mL/kg. For animals receiving the drug as an IV infusion, the appropriate amount of drug solution was injected into a 50-mL bag of saline and was administered as a 20-minute IV infusion. NaHBED monohydrate was put into solution with sterile normal saline at a concentration of 32.4 or 64.9 mg/mL (75 and 150 μmol/kg, respectively) and was given SC to the monkeys at a volume of 1 mL/kg. For IV administration at a dose of 50 or 75 μmol/kg, the drug was dissolved in distilled water and was administered as an IV push at a volume of 1 mL/kg. The drug for the animals dosed at 150 or 225 μmol/kg was prepared as for the bolus injection, except that the appropriate dose of the drug solution was injected into a 50-mL bag of saline and was given as a 20-minute IV infusion. The NaHBED solutions were sterilized by filtration via a 0.2-μm pore size syringe filter.

Calculation of iron chelator efficiency

The efficiency of each chelator was calculated on the basis of a 1:1 ligand-iron complex as described in previous publications.[32,33]

Iron overloading of beagle dogs

Iron dextran (Iron-Gard100, Boehringer Ingelheim Vetmedica, Kansas City, MO) at a concentration of 100 mg Fe/mL and a dose of 100 mg/kg

(1 mL/kg) was administered IV in 100 mL saline over approximately 30 minutes to young adult (10-12 months old, 8-11 kg) male beagle dogs. The procedure was repeated every 10 to 14 days until a total iron burden of 300 mg/kg was reached. Once iron overloading was completed, the iron pools were allowed to equilibrate for at least 1 month before drug dosing began. The equilibration time to allow for iron redistribution for the dogs was shorter than for the monkeys, 30 days versus 60 days, to take account of the increased rate of iron excretion in the dog.[39] Previous studies in which dogs were iron overloaded via IV transfusion of either red blood cells or saccharated iron oxide and underwent tissue biopsies 1 or 4 months later found that there was little difference histologically between the animals biopsied 30 days after overload or those biopsied 4 months after overload.[40] Although the initial rates of release from the 2 carbohydrate polymers are probably different, the dynamics of the iron distribution in and release from the storage compartments would be expected to be similar.

Canine toxicity studies

In one study, iron-overloaded beagles were given either isotonic saline (50 mL, n = 2) or NaHBED (75 μmol/kg in 50 mL isotonic saline, n = 4) IV once daily as a 20-minute infusion for 14 days. Blood was drawn before drug dosing on days 1 and 8 and immediately before euthanizing the dogs for hematologic studies. A routine urinalysis was also carried out at this time. One control and 2 treated animals were euthanized on day 15 (24 hours after drug); the remaining 3 dogs were killed 48 hours after drug administration. On euthanizing, the following tissues were harvested for histologic evaluation: liver, kidney, spleen, lung, right and left atria, right and left ventricles, pericardium, tongue, lymph node, urinary bladder, prostate, mandibular salivary gland, adrenal glands, skin, diaphragm, stomach, duodenum, trachea, right and left papillary muscles, gallbladder, thyroid, small intestine, pancreas, thymus, esophagus, testicle, thigh muscle, jugular vein, cecum, colon, fat, aorta, and rib.

In another experiment, normal dogs (not iron overloaded, 1 year old, n = 2/group) were given NaHBED as a SC bolus at a concentration of 25% (wt/vol) at a dose of 75, 150, or 300 μmol/kg (32.5, 65, or 130 mg/kg) in distilled H_2O. The volume of injection was 1.3, 2.6, or 5.2 mL/10 kg for the 75, 150, and 300 μmol/kg doses, respectively. The drug was administered SC every other day for 14 days (7 doses); the NaHBED injection site was rotated between the right shoulder and left flank such that approximately 96 hours elapsed between injections at the same site. Saline was injected on a similarly rotating basis between the left shoulder and right flank such that each dog served as its own control. Blood was drawn for hematologic and biochemical analyses before drug dosing began, on day 7, and immediately before the animal was euthanized. A routine urinalysis was also carried out at this time. On euthanizing, either 24 or 48 hours after drug administration, the same tissues as those listed above were removed for histopathologic analysis. In addition, skin samples from the drug injection sites and saline injection sites were taken for histologic evaluation.

Assessment of local tolerability in rodents

The general procedure was as follows. The rats (n = 4/group) were anesthetized with sodium pentobarbital (55 mg/kg intraperitoneally) and were given additional anesthetic as needed to keep the animals immobile. The injection sites were the flanks because there is better circulation and much less SC fat in the flanks than in the shoulder area. Skin sections on the flanks were clipped and prepped with an alcohol pad. The drug solutions were sterilized via filtration through a 0.2-μm pore size syringe filter and administered to the rats via a 27-gauge needle. For the infusions, the needle was taped into place for the duration of the infusion. Two rats from each group were killed 48 hours after drug administration, and the remaining 2 were euthanized 1 week after drug administration. Skin sections were removed, examined grossly for signs of irritation, and sent out for histopathologic analysis.

In the SC bolus studies, a single injection was administered to male Sprague-Dawley rats (400 g, 100 μL/ animal) The rats were anesthetized, the skin was prepped, and a 27-gauge needle was used as above. The animals were divided as follows: group 1 received 100 μL isotonic saline; group 2 was given 100 μL 10% (wt/vol) NaHBED in distilled H_2O; group 3 received 100 μL 15% (wt/vol) NaHBED in distilled H_2O, and group 4 was given 100 μL 20% (wt/vol) NaHBED in distilled H_2O.

In the infusion experiments, the rats (400 g) received the drug as a 5-hour SC infusion (100 μL/animal at 20 μL/h). The infusions were delivered using Cadd-Micro Ambulatory Infusion Pumps, model 21-5900 (Deltec, Minneapolis, MN). The animals were anesthetized as described above, and the drug was infused via a 27-gauge needle taped into place. The control rats received saline only (100 μL) as a 5-hour SC infusion. The other rats received NaHBED at 10%, 15%, or 20% concentrations (wt/vol in distilled H_2O) as a 5-hour SC infusion.

Rodent blood pressure and heart rate studies

The basic procedure has been described in an earlier publication.[41] Briefly, normotensive adult male Sprague-Dawley rats (400 g) were anesthetized with sodium pentobarbital (55 mg/kg intraperitoneally). After the neck and shoulder areas were prepared for surgery, an incision was made into the neck area. The proximal end of the carotid artery was tied off with 3-0 silk; the other end was clamped. Once a piece of polyethylene-50 tubing was inserted approximately 3 cm into the artery and tied into place, the tubing was skin tunneled to the shoulder area. The jugular vein was cannulated using a similar procedure. Both of the tubings were flushed with heparin (100 U/mL); a stylet was inserted.

Approximately 24 hours after the surgery, the animals were placed into individual cages and were connected to the blood pressure transducer (AD Instruments, Milford, MA) via the carotid catheter. The blood pressure transducer and the MacLab Bio Amplifier were in turn connected to a Mac Bridge 4 (AD Instruments), a computer-controlled transducer interface. The data were then transmitted to the MacLab 4e data acquisition system powered by a Macintosh Quadra 650. The MacLab Chart program was used to display and analyze the data.

After a half-hour stabilization period, 0.5 mL saline was administered as an IV bolus via the jugular vein catheter, and the blood pressure and heart rate were monitored for 5 minutes. Then, the compound of interest was administered IV at a dose of 300 μmol/kg in a 0.5-mL volume of distilled H_2O as a bolus (n = 5 for both drugs). Once the drug administration was completed, additional saline was given to make sure that all of the drug had been delivered. The blood pressure and heart rate readings were taken for 1 hour after drug administration, during which the animals were unrestrained.

Rats receiving the drug as a 20-minute IV infusion (n = 5 for HBED, n = 4 for DFO) were allowed to acclimate as above. Due to the dead space of the tubing, the infusion pump was allowed to run for approximately 4 minutes before starting the timer. The drug was then given for an additional 20 minutes and the pump was stopped; no additional saline was given as a flush. Blood pressure and heart rate were monitored during the infusion and for 1 hour thereafter.

Statistical analysis

Data are presented as the mean ± SEM. For comparisons of the means of 2 groups, the 2-sample *t* test (without the assumption of equality of variances) was used for analyzing the primate and rodent data. All tests were one-tailed, and a significance level of $P < .05$ was used.

Results

Comparisons of chelator-induced iron excretion in *C apella* monkeys given SC versus IV NaHBED and those given SC versus IV DFO

These studies (Table 1) were carried out in iron-overloaded (~500 mg/kg) primates.[35] In brief, DFO was administered either SC at doses of 75 and 150 μmol/kg or as a 20-minute IV infusion at doses of 75 and 150 μmol/kg. Three methods of administration of NaHBED were investigated: SC bolus at doses of 75 and 150 μmol/kg, IV bolus at doses of 50 and 75 μmol/kg, and 20-minute IV infusion at doses of 150 and 225 μmol/kg.

Table 1. Comparison of efficiencies and net iron balance of IV and SC HBED versus those of IV and SC DFO in *C apella* primates

Drug	Route	Dose (μmol/kg)	Dose (mg/kg)	n	Efficiency (%)	Induced Fe (μg/kg)	Fe balance (μg/kg)*
DFO	SC bolus	75	50	4	5.0 ± 2.6	213 ± 112	−60 ± 135
DFO†	SC bolus	150	100	6	5.1 ± 1.3	435 ± 115	−278 ± 185
DFO	20-min IV inf	75	50	5	5.6 ± 1.6	237 ± 67	−22 ± 197
DFO	20-min IV inf	150	100	5	3.9 ± 0.8	332 ± 66	−168 ± 51
HBED‡	SC bolus	75	32.4	4	14.2 ± 2.2	597 ± 91	−524 ± 84
HBED§	SC bolus	150	64.9	5	13.6 ± 4.5	1139 ± 383	−899 ± 365
HBED	IV bolus	50	21.6	5	12.1 ± 2.5	338 ± 68	−195 ± 114
HBED	IV bolus	75	32.4	4	11.5 ± 1.3	482 ± 54	−407 ± 73
HBED	20 min IV inf	150	64.9	4	7.7 ± 0.8	644 ± 65	−408 ± 77
HBED	20 min IV inf	225	97.3	5	6.3 ± 1.0	798 ± 126	−570 ± 248

*Net iron balance = dietary iron intake − (urinary iron + fecal iron). Animals in a negative iron balance are excreting more iron than they are absorbing. To maintain iron balance, 250 to 400 μg Fe/kg/day[42] = 1750 to 2800 μg Fe/kg/week must be cleared.

†Previously published.[32]

‡Derived from our previously published study[33] in which the monkeys were given 75 μmol/kg as an SC bolus in saline on days 0, 2, and 4; the day +1 to day +2 versus day −3 to day 0 iron balance figures are shown.

§Previously published.[33]

Deferoxamine administered SC at a dose of 75 μmol/kg induced the excretion of 213 ± 112 μg Fe/kg; the efficiency was 5.0% ± 2.6%. Increasing the dose to 150 μmol/kg resulted in the excretion of approximately twice the amount of iron, 435 ± 115 μg/kg, an efficiency of 5.1% ± 1.3% ($P > .4$). DFO given IV at a dose of 75 μmol/kg induced the excretion of 237 ± 67 μg Fe/kg and had an efficiency of 5.6% ± 1.6%. Increasing the dose to 150 μmol/kg resulted in the excretion of 332 ± 66 μg Fe/kg and an efficiency of 3.9% ± 0.8% ($P < .04$). Interestingly, although the efficiency of DFO given either SC or IV at a dose of 75 μmol/kg was similar, 5.0% ± 2.6% versus 5.6% ± 1.6% ($P > .3$), this was not the case when the dose was increased to 150 μmol/kg. DFO given SC at 150 μmol/kg resulted in an iron clearing efficiency of 5.1% ± 1.3%, but the same dose given as an IV infusion resulted in an efficiency of 3.9% ± 0.8% ($P < .05$).

At a dose of 75 μmol/kg SC, NaHBED induced the excretion of 597 ± 91 μg Fe/kg, an efficiency of 14.2% ± 2.2%. Doubling the dose to 150 μmol/kg SC resulted in the excretion of almost twice as much iron, 1139 ± 383 μg/kg and an efficiency of 13.6% ± 4.5% ($P > .3$). The efficiencies of the drug administered as an IV bolus at doses of 50 and 75 μmol/kg were also similar, 12.1% ± 2.5% and 11.5% ± 1.3% ($P > .3$), with a corresponding iron excretion of 338 ± 68 μg/kg and 482 ± 54 μg/kg, respectively. Finally, the efficiency of NaHBED given SC at a dose of 75 μmol/kg was greater than that for the same dose given as an IV bolus, 14.2% ± 2.2% versus 11.5% ± 1.3%, respectively ($P < .05$). When given as a 20-minute IV infusion, an increase in the dose of NaHBED from 150 to 225 μmol/kg resulted in the excretion of more iron (from 644 ± 65 to 798 ± 126 μg/kg) but a decline in efficiency (from 7.7% ± 0.8% to 6.3% ± 1.0%).

Systemic toxicity of NaHBED in dogs

Four beagles that had been iron loaded to a level of 300 mg Fe/kg were given an IV dose of 75 μmol/kg NaHBED in 50 mL isotonic saline as a 20-minute infusion once daily for 14 days. Two additional dogs, also iron loaded to a level of 300 mg Fe/kg, served as saline-treated controls. On euthanizing, 24 or 48 hours after dosing and extensive histopathologic analysis, the most significant finding was the accumulation of hemosiderin in the macrophages of the liver, spleen, and lymph nodes of both test and control animals. There was no systemic toxicity that could be attributed to the NaHBED.

A second systemic toxicity trial was carried out in dogs using SC administration of NaHBED. These non–iron-overloaded dogs (n = 2/dose) were given NaHBED at doses of 75, 150, or 300 μmol/kg. The drug was injected as an SC bolus every other day into 1 of 2 sites on a rotating basis such that about 96 hours elapsed between injections at the same site. On euthanizing, either 24 or 48 hours after drug delivery, histopathologic analysis did not reveal any drug-related abnormalities beyond those in the skin. The skin at the sites that were injected with NaHBED did present with mild to significant reactions. The descriptions of the reactions ranged from early, focally extensive fibroplasia and mild, superficial subcutis to panniculitis (both lymphohistiocytic and neutrophilic), which was subacute and focally extensive, and moderate to severe deep subcutis. The descriptions of the skin from the sites injected with saline included early fibroplasia, which ranged from diffuse, moderate, and superficial to focally extensive, and deep subcutis; one site presented with panniculitis (both lymphohistiocytic and neutrophilic).

Recall that the drug was administered to the dogs SC at a concentration of 25% (wt/vol) and a volume of injection of 1.3, 2.6, or 5.2 mL/10 kg for the 75-, 150-, and 300-μmol/kg doses, respectively. In addition, there did appear to be a graded response, with the animals receiving higher volumes having more local irritation than those receiving lower volumes. This observation is in keeping with a surface-to-volume problem, in which the surrounding tissues could not supply sufficient fluid to compensate for the hypertonicity of the larger volumes of solution administered. The local irritation at the injection sites observed in the dogs was explored further in a rodent model.

Studies of local irritation at NaHBED injection sites in rodents

The results in dogs and preliminary experiments in rodents suggested to us that the hypertonicity of the 25% (wt/vol) solution used might be responsible for the local irritation observed. Accordingly, groups of 4 rodents were given a 100-μL SC bolus of (1) isotonic saline, (2) 10% NaHBED in distilled H_2O, (3) 15% NaHBED in distilled H_2O, or (4) 20% NaHBED in distilled H_2O. Animals were also administered the same volume (100 μL) as a 5-hour SC infusion (ie, at a rate of 20 μL/h). These groups (n = 4) were given (1) isotonic saline, (2) 10% NaHBED in distilled H_2O, (3) 15% NaHBED in distilled H_2O, or (4) 20% NaHBED in distilled H_2O. Note that a 300-g rat receiving 100 μL of the drug

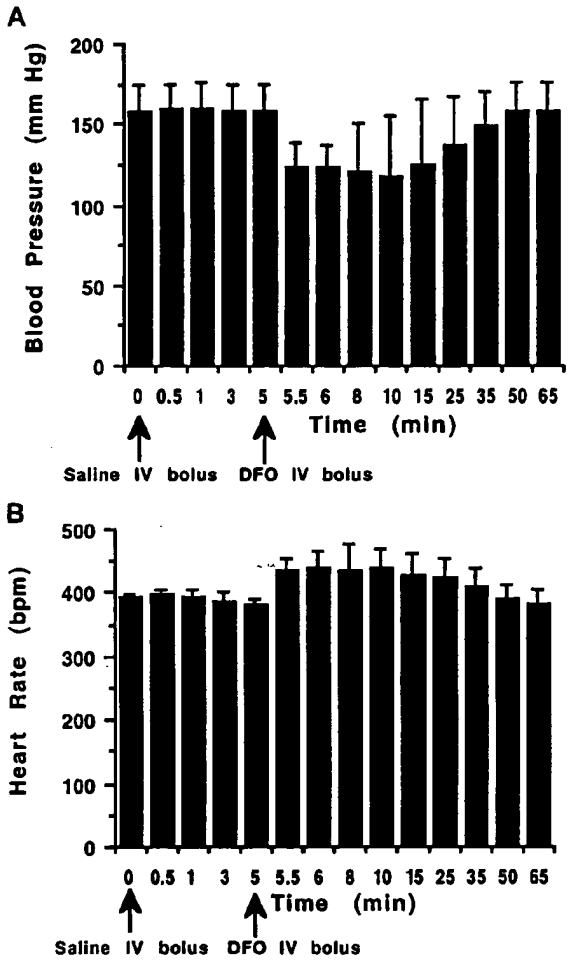


Figure 2. DFO by IV bolus. The effect of IV bolus administration of DFO (300 μmol/kg) on the blood pressure (A, mm Hg) and heart rate (B, beats/min) is shown for normotensive rats (n = 5). For panel A, $P < .001$ for $t = 5.5$ to 15 minutes; $P < .005$ for $t = 25$ minutes. For panel B, $P < .001$ for $t = 5.5$ to 35 minutes.

solution would be receiving a volume of drug solution roughly comparable to administration of 20 mL of the drug solution to a 60-kg person. Again, 2 animals from each group were killed 48 hours after dosing and 2 more 7 days after dosing. The histopathologic descriptors for both bolus and infusion saline controls at 48 hours and 7 days included endothelial hypertrophy, minimal inflammation, scattered mast cells, and subcutis. With the exception of the rats treated with 20% NaHBED as a SC bolus, in which mild panniculitis was noted in one each of the rodents killed 48 hours or 1 week after dosing, all of the test animals presented with essentially the same histopathology as the control animals. Therefore, we have demonstrated that it is possible to prevent NaHBED-related irritation by either giving the drug as a slow SC infusion or as a SC bolus at concentrations of 15% wt/vol or less.

Impact of NaHBED and DFO on blood pressure and heart rate in rodents

Owing to the profound impact that DFO administered IV can have on blood pressure and cardiac function,[26] the manufacturer recommends that the drug be given by this route at doses not to exceed 15 mg/kg per hour (22 μmol/kg per hour), even under circumstances of cardiovascular collapse from acute iron toxicity. On consideration of this, we elected to compare the impact of DFO and NaHBED administered IV on blood pressure and heart rate in normotensive rodents. The chelators were administered at a dose of 300 μmol/kg in a 0.5-mL volume either by IV bolus (n = 5 for both drugs) or by IV infusion over a 20-minute period (n = 5 for NaHBED, n = 4 for DFO).

When rodents were given 300 μmol/kg DFO as an IV bolus, there was a 25% decrease in blood pressure that did not return to baseline levels until 35 minutes after drug delivery ($P < .001$ for $t = 5.5$ to 15 minutes and $P < .005$ for $t = 25$ minutes, Figure 2A). The heart rate in these animals also increased by 16% and likewise did not return to predrug levels until more than 35 minutes after dosing ($P < .001$ for $t = 5.5$ to 35 minutes, Figure 2B). When DFO was administered at 300 μmol/kg as a 20-minute IV infusion, no effect on either blood pressure or heart rate (Figure 3) was recorded. In contrast, there was no effect on either blood pressure or heart rate in rats given the same dose of NaHBED as an IV bolus (Figure 4) or as a 20-minute IV infusion (Figure 5). The lack of an effect of NaHBED administered IV on either blood pressure or heart rate makes NaHBED an attractive therapeutic for the treatment of acute iron poisoning.

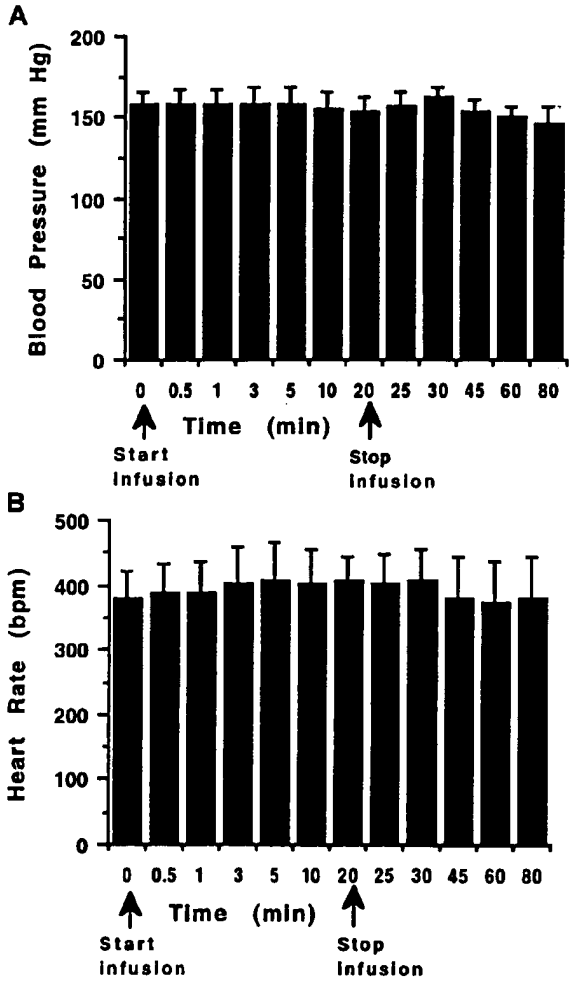


Figure 3. DFO by IV infusion. The effect of IV infusion administration of DFO (300 μmol/kg, 0.5 mL over 20 minutes) on the blood pressure (A, mm Hg) and heart rate (B, beats/min) is shown for normotensive rats (n = 4).

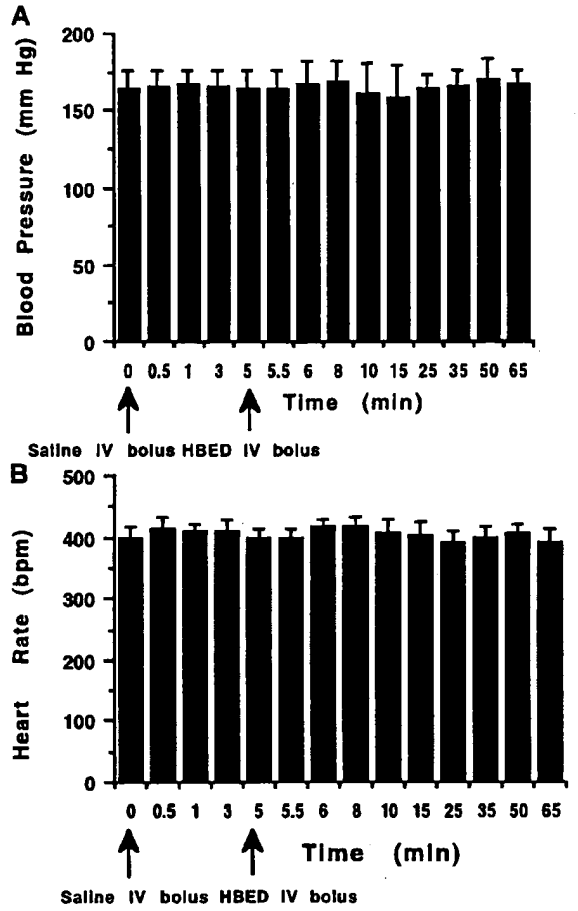


Figure 4. NaHBED by IV bolus. The effect of IV bolus administration of NaHBED (300 μmol/kg) on the blood pressure (A, mm Hg) and heart rate (B, beats/min) is shown for normotensive rats (n = 5).

Discussion

We have continued the preclinical evaluation of the efficacy and safety of NaHBED for the treatment of both chronic transfusional iron overload and of acute iron poisoning with a series of studies in monkeys, rodents, and dogs. First, we extended our previous investigation of the effect of HBED given SC on iron excretion in the iron-loaded *C apella* monkey with comparative studies of NaHBED and DFO after SC and IV administration. To provide a quantitative frame of reference, most patients who are dependent on transfusions receive about 200 to 300 mL blood/kg body weight per year, an amount corresponding to about 250 to 400 μg Fe/kg body weight per day.[42] Clinically, the recommended dose of DFO for the treatment of transfusional iron overload is 75 μmol/kg (50 mg DFO/kg) or less; doses more than 150 μmol/kg (100 mg DFO/kg) are used only in exceptional circumstances because of the increased risk of neurotoxicity and other adverse effects.

As detailed in Table 1, we compared NaHBED and DFO when equimolar amounts were given as a SC bolus injection or as a 20-minute IV infusion and calculated the efficiency of iron chelation, which is expressed as a percent. The observed iron excretion with DFO in the primates after SC injection at doses of 75 and 150 μmol/kg is consistent with the established ability of DFO to control body iron in patients with transfusional iron overload. With SC bolus injection, the efficiency of iron chelation with DFO remained about the same at both doses, approximately 5%. By comparison, the observed iron excretion with NaHBED after SC injection at doses of 75 and 150 μmol/kg was almost 3-fold greater than that seen with an injection of SC DFO. The efficiency of iron chelation with SC injection of NaHBED also remained approximately constant at both doses, about 14%.

When DFO was given as a 20-minute IV infusion at a dose of 75 μmol/kg, the efficiency of chelation, 5.6% ± 1.6%, was about the same as that found with SC injection at the same dose. At the higher dose of 150 μmol DFO/kg, more iron was excreted, but the efficiency of chelation fell to 3.9% ± 0.8% ($P < .04$). This nonlinear dose response, also observed in patients treated with escalating IV doses of DFO,[43] suggests that the chelatable iron pool accessible to DFO with this mode of administration is limited. For comparison, at the equimolar dose of 150 μmol/kg also given as a 20-minute IV infusion, the efficiency of NaHBED, 7.7% ± 0.8%, was almost twice that of DFO ($P < .001$). Nonetheless, the brief IV infusion of 150 μmol NaHBED/kg was only about half as efficient as the same dose of NaHBED administered SC, presumably because of the longer duration of action for this dose provided by the SC route. With a still higher dose of 225 μmol NaHBED/kg, efficiency fell further, to 6.3% ± 1.0% ($P < .01$ versus the 150

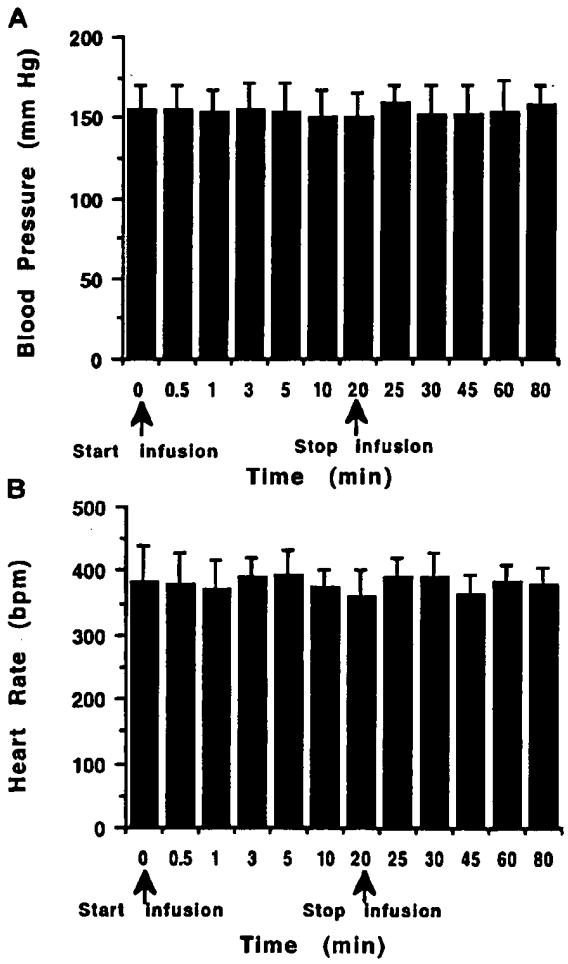


Figure 5. NaHBED by IV infusion. The effect of IV infusion administration of NaHBED (300 μmol/kg) on the blood pressure (A, mm Hg) and heart rate (B, beats/min) is shown for normotensive rats (n = 5).

μmol/kg SC dose). Moreover, at the lower dose of 75 μmol NaHBED/kg, given as an IV bolus, the observed efficiency of chelation, 11.5% ± 1.3%, was slightly lower than that of the same dose of NaHBED given SC, 14.2% ± 2.2% ($P < .05$). Nevertheless, these results suggest that, at the doses of NaHBED likely to be used clinically, 75 μmol/kg or less, the iron excretion with bolus administration by either the SC or IV route will be well within the range of 250 to 400 μg Fe/kg necessary to maintain iron balance and will be 2- to 3-fold greater than that observed with equimolar doses of SC DFO.

We next examined the safety of NaHBED, administered by 20-minute IV infusion at a dose of 75 μmol/kg daily to iron-loaded beagle dogs, and by SC bolus at doses of 75, 150, or 300 μmol/kg as a 25% (wt/vol) solution every other day to beagle dogs without iron overload. No evidence of systemic toxicity was found in either study. In the study of SC administration, more local irritation was found at injection sites with the hypertonic solutions of NaHBED than at those sites with isotonic saline. To determine the source of the irritation at injection sites, we carried out a series of experiments with male Sprague-Dawley rats. In brief, these studies indicated that the tonicity of the solution injected was the factor producing irritation at the injection sites. Irritation could be avoided by using volumes comparable to those that would be used clinically and by using less hypertonic solutions (≤ 15% [wt/vol]).

Finally, because treatment of acute iron poisoning may require rapid chelator infusion, we compared the effects of IV administration of NaHBED or DFO on the blood pressure and heart rate of normotensive Sprague-Dawley rats. Iron salts remain the leading cause of death from accidental poisoning in children in the United States. Although toxicity associated with the ingestion of less than 20 mg/kg elemental iron is generally self-limiting, intake of 20 to 60 mg/kg elemental iron may result in mild to moderate toxicity; the consumption of more than 60 mg/kg elemental iron is potentially life-threatening.[44] Owing to the profound impact that IV DFO can have on blood pressure and cardiac function,[26] the manufacturer recommends that the drug be given by this route to these patients at doses not to exceed 15 mg/kg per hour (22 μmol/kg per hour). In addition, IV DFO should be given only under circumstances of cardiovascular collapse, and even then at doses not to exceed 15 mg/kg per hour for the first 1000 mg. Subsequent IV administration, if needed, must be at a slower rate not to exceed 125 mg/hour. Our results suggest that IV infusion of NaHBED for the treatment of acute iron poisoning will not be subject to these restrictions. In our studies, rapid IV infusion of DFO to normotensive rats promptly produced a substantial, prolonged drop in blood pressure and acceleration in heart rate. By contrast, NaHBED had no significant effect. NaHBED could thus potentially be administered much more rapidly and in greater amounts than DFO in the treatment of acute iron poisoning.

Our results have provided additional evidence for the safety and tolerability of NaHBED when administered either in the manner that would be used chronically, for the treatment of iron overload, or acutely, for the treatment of iron poisoning. Comparative studies of iron excretion in the iron-loaded *C apella* monkey have found that NaHBED is 2 to 3 times more efficient as an iron chelator than DFO after either SC or IV administration. Overall, these results indicate the need for prompt completion of the preclinical evaluation of parenteral NaHBED in preparation for studies of iron balance in human volunteers. NaHBED may provide an alternative to DFO for the treatment of both chronic transfusional iron overload and of acute iron poisoning.

Acknowledgments

The authors would like to thank Katie Ratliff-Thompson, Tammy Fannin, and Michael A. Slusher for their technical assistance and Dr Eileen Eiler-McManis for her editorial comments.

References

1. Olivieri NF, Brittenham GM. Iron-chelating therapy and the treatment of thalassemia. Blood. 1997;89:739-761.
2. Weatherall DJ. The treatment of thalassemia—slow progress and new dilemmas. N Engl J Med. 1993;329:877-879.
3. Cao A, Galanello R, Rosatelli MC, Argiolu F, De Virgiliis S. Clinical experience of management of thalassemia: the Sardinian experience. Semin Hematol. 1996;33:66-75.
4. Gabutti V, Piga A. Results of long-term iron-chelating therapy. Acta Haematol. 1996;95:26-36.
5. Giardina PJ, Grady RW. Chelation therapy in beta-thalassemia: the benefits and limitations of desferrioxamine. Semin Hematol. 1995;32:304-312.
6. Wolfe L, Olivieri N, Sallan D, et al. Prevention of cardiac disease by subcutaneous deferoxamine in patients with thalassemia major. N Engl J Med. 1985;312:1600-1603.
7. Bickel H, Hall GE, Keller-Schierlein W, et al. Metabolic products of actinomycetes, XXVII: constitutional formula of ferrioxamine B. Helv Chim Acta. 1960;43:2129-2138.
8. Lee P, Mohammed N, Marshall L, et al. Intravenous infusion pharmacokinetics of desferrioxamine in thalassaemic patients. Drug Metab Dispos. 1993;21:640-644.
9. Pippard MJ, Callender ST, Finch CA. Ferrioxamine excretion in iron-loaded man. Blood. 1982;60:288-294.
10. Pippard MJ. Desferrioxamine-induced iron excretion in humans. Bailleres Clin Haematol. 1989;2:323-343.
11. Porter JB, Huehns ER. The toxic effects of desferrioxamine. Bailleres Clin Haematol. 1989;2:459-474.
12. Porter JB, Jaswon MS, Huehns ER, East CA, Hazell JW. Desferrioxamine ototoxicity: evaluation of risk factors in thalassaemic patients and guidelines for safe dosage. Br J Haematol. 1989;73:403-409.
13. Koren G, Kochavi-Atiya Y, Bentur Y, Olivieri NF. The effects of subcutaneous deferoxamine administration on renal function in thalassemia major [letter]. Int J Haematol. 1992;54:371.
14. Athanasiou A, Shepp MA, Necheles TF. Anaphylactic reaction to desferrioxamine [letter]. Lancet. 1977;2:616.
15. Shalit M, Tedeschi A, Miadonna A, Levi-Schaffer F. Desferal (desferrioxamine)—a novel activator of tissue-type mast cells. J Allergy Clin Immunol. 1991;88:854-860.
16. Bousquet J, Navarro M, Robert G, Aye P, Michel FB. Rapid desensitization for desferrioxamine anaphylactoid reactions. Lancet. 1983;2:859-860.
17. Miller KB, Rosenwasser LJ, Bessette JAM, Beer DJ, Rocklin RE. Rapid desensitisation for desferrioxamine anaphylactic reaction [letter]. Lancet. 1981;1:1059.
18. Olivieri NF, Buncic JR, Chew E, et al. Visual and auditory neurotoxicity in patients receiving subcutaneous deferoxamine infusions. N Engl J Med. 1986;314:869-873.
19. Tenenbein M, Kowalski S, Sienko A, Bowden DH, Adamson IYR. Pulmonary toxic effects of continuous deferoxamine administration in acute iron poisoning. Lancet. 1992;339:699-701.
20. Brill PW, Winchester P, Giardina PJ, Cunningham-Rundles S. Desferrioxamine-induced bone dysplasia in patients with thalassemia major. Am J Roentgenol. 1991;156:561-565.
21. DeVirgiliis S, Congia M, Frau F, et al. Deferoxamine-induced growth retardation in patients with thalassemia major. J Pediatr. 1988;113:661-669.
22. Nouel O, Voisin PM, Vaucel J, Dartois Hoguin M, Le Bris M. *Yersinia enterocolitica* septicemia associated with idiopathic hemochromatosis and deferoxamine therapy. A case. Presse Med. 1991;20:1494-1496.
23. Cohen AR, Mizanin J, Schwartz E. Rapid removal of excess iron with daily, high-dose intravenous chelation therapy. J Pediatr. 1989;115:151-155.
24. Olivieri NF, Berriman AM, Tyler BJ, et al. Reduction in tissue iron stores with a new regimen of continuous ambulatory intravenous deferoxamine. Am J Hematol. 1992;41:61-63.
25. Lombardo T, Ferro G, Frontini V, Percolla S. High-dose intravenous desferrioxamine (DFO) delivery to four thalassemic patients allergic to subcutaneous DFO administration. Am J Hematol. 1996;51:90-92.
26. Whitten CF, Gibson GW, Good MH, Goodwin JF, Brough AJ. Studies in acute iron poisoning. I: Desferrioxamine in the treatment of acute iron poisoning: clinical observations, experimental studies, and theoretical considerations. Pediatrics. 1965;36:322-335.

27. Freedman MH, Grisaru D, Olivieri N, MacLusky I, Thorner PS. Pulmonary syndrome in patients with thalassemia major receiving intravenous deferoxamine infusions. Am J Dis Child. 1990;144:565-569.
28. Ioannides AS, Pansiello JM. Acute respiratory distress syndrome in children with acute iron poisoning: the role of intravenous desferrioxamine. Eur J Pediatr. 2000;159:158-159.
29. L'Eplattenier F, Murase I, Martell AE. New multidentate ligands, VI: chelating tendencies of *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid. J Am Chem Soc. 1967; 89:837-843.
30. Brittenham GM. Development of iron-chelating agents for clinical use. Blood. 1992;80:569-574.
31. Porter JB. Evaluation of new iron chelators for clinical use. Acta Haematol. 1996;95:13-25.
32. Bergeron RJ, Wiegand J, Brittenham GM. HBED: a potential alternative to deferoxamine for iron-chelating therapy. Blood. 1998;91:1446-1452.
33. Bergeron RJ, Wiegand J, Brittenham GM. HBED: the continuing development of a potential alternative to deferoxamine for iron-chelating therapy. Blood. 1999;93:370-375.
34. Bergeron RJ, Streiff RR, Creary EA, et al. A comparative study of the iron-clearing properties of desferrithiocin analogues with desferrioxamine B in a *Cebus* monkey model. Blood. 1993;81:2166-2173.
35. Bergeron RJ, Streiff RR, Wiegand J, et al. A comparison of the iron-clearing properties of 1,2-dimethyl-3-hydroxypyrid-4-one, 1,2-diethyl-3-hydroxypyrid-4-one, and deferoxamine. Blood. 1992;79:1882-1890.
36. Wood JK, Milner PF, Pathak UN. The metabolism of iron-dextran given as a total-dose infusion to iron deficient Jamaican subjects. Br J Haematol. 1968;14:119-129.
37. Bergeron RJ, Streiff RR, Wiegand J, et al. A comparative evaluation of iron clearance models. Ann N Y Acad Sci. 1990;612:378-393.
38. Bergeron RJ, Wiegand J, Weimar WR, et al. Desazadesmethyldesferrithiocin analogues as orally effective iron chelators. J Med Chem. 1999;42:95-108.
39. Finch CA, Ragan HA, Dyer IA, Cook JD. Body iron loss in animals. Proc Soc Exp Biol Med. 1978;159:335-338.
40. Brown EB, Rubenia D, Smith DE, Reynafarje C, Moore C. Studies in iron transportation and metabolism X. Long-term iron overload in dogs. J Lab Clin Med. 1957;50:862-893.
41. Bergeron RJ, Wiegand J, Sninsky CA, Katovich MJ. The impact of polyamine analogues on the blood pressure of normotensive and hypertensive rats. Clin Exp Hypertension. 1995;17:1197-1217.
42. Brittenham GM. Pyridoxal isonicotinoyl hydrazone (PIH): effective iron chelation after oral administration. Ann N Y Acad Sci. 1990;612:315-326.
43. Propper RD, Shurin SB, Nathan DG. Reassessment of the use of desferrioxamine B in iron overload. N Engl J Med. 1976;294:1421-1423.
44. Litovitz TL, Holm KA, Bailey KM, Schmitz BF. 1991 annual report of the American Association of Poison Control Centers national data collection system. Am J Emerg Med. 1992;10:452-505.

Appendix B

Drug Toxicity and Metabolism in Pediatrics

Editor

Sam Kacew

Professor
Department of Pharmacology
University of Ottawa
Ottawa, Ontario, Canada

CRC Press, Inc.
Boca Raton, Florida

Library of Congress Cataloging-in-Publication Data
Drug toxicity and metabolism in pediatrics.

Includes bibliographical references.
1. Pediatric pharmacology. 2. Pediatric toxicology.
3. Drugs--Side effects. 4. Drugs--Metabolism.
5. Children--Metabolism. I. Kacew, Sam.
RJ560.D7889 1990 615'.704 89-17329
ISBN 0-8493-4564-2

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida 33431.

International Standard Book Number 0-8493-4564-2

Library of Congress Card Number 89-17329
Printed in the United States

Chapter 16

IRON POISONING

Theodore G. Tong and William Banner, Jr.

TABLE OF CONTENTS

I. INTRODUCTION

Iron-containing products remain a continuing toxicologic hazard to children. In the 1987 report of the American Association of Poison Control Centers' National Data Collection System,[1] 17,145 cases of acute iron ingestion had been reported to 63 participating regional poison centers involved in data collection. Data gathered by the statewide Arizona Poison Control System[33] show ferrous sulfate tablets and vitamins containing iron remain among the top ten substances ingested by children younger than 5 years of age. Widespread availability, the large number of tablets prescribed, a failure of parents and the general public to recognize the potential lethality of iron intoxication, and the failure of safety closures have all been suggested as contributors to the continuing risk of accidental ingestion of these products.[2] Despite the frequency of iron intoxication and the many articles suggesting various therapies for iron intoxication, the management and determination of severity during acute iron poisoning remains, for clinicians, an area of confusion and much controversy.

II. TOXIC EFFECTS OF IRON

The pathophysiology of iron intoxication was initially described by Covey in 1954.[3] The clinical features of this condition can be characterized by five stages:

1. Gastrointestinal (GI) toxicity,
2. Apparent improvement and stability
3. Circulatory shock
4. Hepatic necrosis
5. Gastric scarring

The time course, severity, and when these stages occur are related to the amount of iron ingested and the response to ongoing treatment.

A. GASTROINTESTINAL TOXICITY

The first obvious signs of toxicity in acute iron ingestions are gastrointestinal. These effects include vomiting, rapid onset of diarrhea, colicky abdominal pain, and GI hemorrhage. The mechanism of this toxicity is not clearly defined. Symptoms may appear as early as 30 min, but are usually present within 6 h following ingestion. In the routine absorption of dietary iron, the transport of ferrous sulfate involves a complex energy-dependent transport system. This saturable, carrier-mediated uptake process is dependent upon binding of iron in the lumen of the GI tract and is the rate-limiting step for iron absorption. Toxic amounts of iron in solution are rapidly absorbed in the large and small bowel in a concentration-dependent fashion, suggesting that a saturable transport system is not involved at high concentrations. Thus, in the acute overdose situation, it is presumed that the normal mechanisms of absorption have been exceeded and that absorption is a passive, first-order process.

Iron acutely and directly damages the GI tract. Histologically, the gastric and small intestinal mucosa appear to bear the burden of damage. Deposition of iron can be demonstrated in mucosal collagen and basement membranes and in cell nuclei and tissues of the vascular supply to the small bowel. Coagulative necrosis with platelet aggregation is observed at these sites. In many ways, there are stiking similarities in the amount and extent of damage that take place in the GI tract when acute iron and corrosive ingestion are compared. In the absence of frank hemorrhage, the GI effects of iron may lead to systemic hypovolemia by causing "third spacing" of fluid into the small bowel.[4]

1. Apparent Improvement and Stability

Given the acute effects of iron on the GI tract and the rapid loss of fluid volume, it

seems unlikely that severe iron intoxication could be associated with an absence of obvious symptoms. This poorly described second stage of iron poisoning begins as early as 3 to 4 h after the ingestion, lasting as long as 48 h. Poor tissue perfusion, mild hyperventilation (secondary to acidosis), oliguria, and continued GI symptoms should suggest the presence of a severe ingestion. In cases of mild severity, recovery is complete with only supportive therapy given during the quiescent period. If the iron poisoning is moderate to severe, the patients's clinical status will worsen and the course quickly evolves into the third stage.

B. CIRCULATORY SHOCK

The third stage of iron poisoning is characterized by acute circulatory shock. The mechanisms of shock in iron poisoning are multifactorial and therefore treatment must be directed at correcting the various causes. These factors all contribute to profound hypovolemia and hypoperfusion, leading to shock. In animal models where this has been studied,[4,5] certain consistent factors have been found. Following the acute administration of lethal doses of ferrous sulfate, there is a dramatic decrease in circulating plasma volume, as reflected in increases in the hematocrit. Cardiac output is lowered on the basis of a decreased filling pressure. In addition to fluid losses, this decrease in effective filling pressure may be caused from metabolic acidosis and venous pooling by a direct loss of vascular tone.

An explanation given for the metabolic acidosis is it results from the conversion from the ferrous to the ferric ionic state following absorption. Studies have demonstrated that direct addition of iron in the ferrous state to blood may directly decrease bicarbonate.[6] The inhibitory effects of iron on complex oxidative metabolism at the cellular level may also result in increasing lactic acid concentrations.[6] Low cardiac output and high systemic resistance are the clinical presentations seen in the acutely iron-intoxicated infant. Such infants present in a tachycardic and extremely pale condition, with cold extremities and decreased central venous pressures.

The coagulopathy and resultant bleeding associated with iron intoxication may contribute to hypovolemia. The basis for this abnormality is probably secondary to hypoperfusion and intravascular damage. Free iron in the circulation inhibits the action of thrombin on fibrinogen and adversely affects coagulation.[7] Additional evidence points to a diminished ability to produce thrombin in the presence of high concentrations of iron.[8,9]

Shock from iron intoxication rquires early and aggressive treatment. Restoration of intravascular volume is critical before the effects of hypoperfusion and ischemia to tissue become irreversible. The need for prompt recognition of the intravascular volume status of the patient and the potential for GI hemorrhage and primary disturbances of coagulation make it imperative for the clinician to monitor more than simply blood pressure. By observing for tachycardia, poor perfusion of skin, oliguria, and acidosis, early intervention can be undertaken to reverse the progression of the adverse circulatory effects.

C. HEPATIC NECROSIS

During stage four, symptoms and findings are related to acute hepatic injury. The liver is a target organ for damage by direct uptake of iron in intoxication. Iron can be taken up directly by the reticuloendothelial system of the liver and spleen and it exerts a toxic effect on the mitochondria of the liver cell. Fatty degeneration mitochrondrial and portal injuries are changes seen in the liver. Fortunately, this is an extremely rare clinical manifestation of iron toxicity.

D. GASTRIC SCARRING

Following an acute corrosive insult to the GI tract, the healing process may result in areas of stenosis and stricture formation in both the stomach outlet and the small bowel. The late consequences of iron poisoning occur rarely. They may present as late as 2 to 6 weeks after the inciting event.

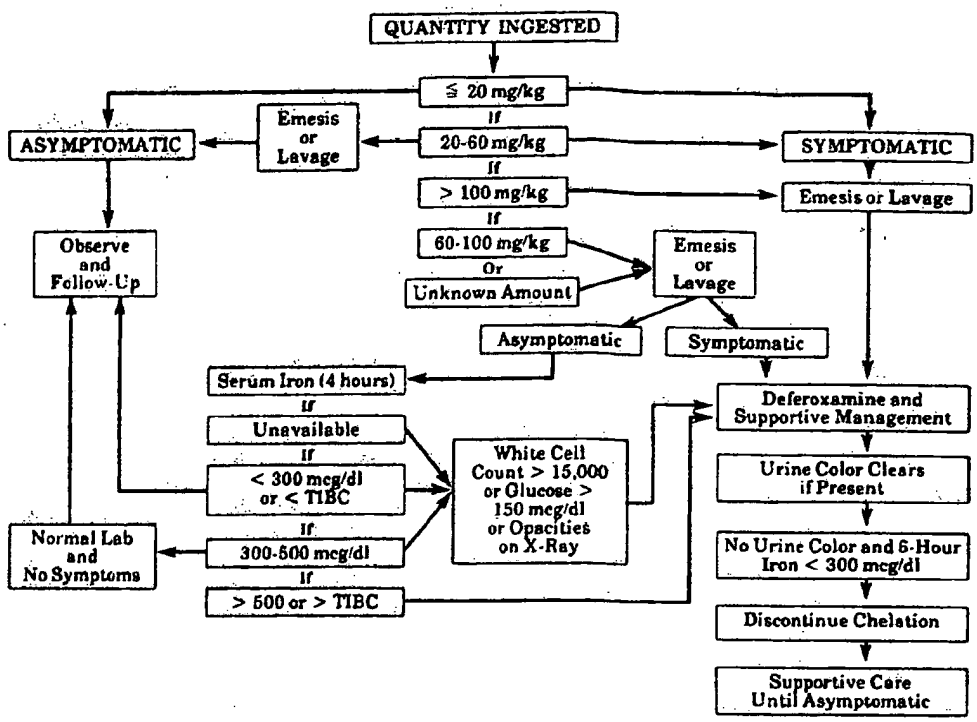


FIGURE 1. Steps to management of acute iron poisoning. (From Banner, W. and Tong, T. G., *Pediatr. Clin. North Am.*, 3, 402, 1986. With permission.)

III. APPROACHES TO MANAGEMENT

The large number of exposures to iron-containing products and the relatively infrequent mortality suggest that a wide range of severity exists in acute iron intoxication. Some guidelines must be used to assure the appropriate triage of the iron-intoxicated patient to provide an appropriate level of intervention. In addition, the appropriate use of laboratory tests and X-ray studies is demanded by restraints on treatment and hospitalization costs. The challenge involved with the management of iron poisoning is to recognize the severity of the occasion and to intervene by providing chelation therapy and supportive care in situations wherever indicated.

A. TRIAGE

Attempting to base protocols for triage on a single factor, such as the ingested dose, overlooks the complexities of human poisoning. A multifactorial approach to iron intoxication that includes the maximal estimated dose, the clinical status of the patient, and the serum iron level determination should be attempted. Figure 1 presents an algorithm for an approach to the triage of the iron-intoxicated patient.

Whenever calculating milligrams per kilogram doses of iron, it should be kept in mind that the *elemental* iron per dosage unit is only a small fraction of the total milligram weight of a capsule or tablet. Table 1 shows the amount of the elemental iron for a number of popular iron-containing products. Our approach is to always take into account the worst possible case whenever the milligrams per kilogram per dose is used to assess severity. When considering the number of possible tablets ingested, estimates of how full the bottle was or number of tablets previously used may yield misleading results. In general, we assume that the maximal number of tablets missing from a bottle is the amount that the

TABLE 1
Iron Content of Common Iron-Containing Products

Product	Total elemental iron per dosage unit (mg/tablet)
Ferrous sulfate, 325 mg	65 mg
Ferrous gluconate, 325 mg	40 mg
Ferrous fumarate, 325 mg	105 mg
Ferrous fumarate, 200 mg	66 mg
Chocks® plus iron	18 mg
Chocks Bugs Bunny® plus iron	18 mg
Feosol® elixir (sulfate)	44 mg/5 ml
Fergon® elixir (gluconate)	35 mg/5 ml
Fer-In-Sol® capsules (sulfate)	60 mg
Fer-In-Sol® drops	15 mg/0.6 ml
Fer-In-Sol® syrup	18 mg/5 ml
Filibon® capsules (fumarate)	30 mg
Flinstones plus iron	18 mg
Monster vitamins with iron (fumarate)	12 mg
Natabec (sulfate)	30 mg
Natalins® Rx (fumarate)	60 mg
Natalins® (fumarate)	45 mg
One A Day® plus iron	18 mg
One A Day® plus minerals	18 mg
Poly-Vi-Flor® drops with iron (sulfate)	10 mg/ml
Poly-Vi-Sol® drops with iron (sulfate)	10 mg/ml
Poly-Vi-Flor® tablets with iron (fumarate)	12 mg
Poly-Vi-Sol® tablets with iron (fumarate)	12 mg
Spider-man® vitamins with iron	18 mg
Stuart formula tablets (fumarate)	60 mg
Stuartinic® tablets (fumarate)	100 mg
Theragram M® tablets (carbonate)	12 mg
Tri-Vi-Sol® drops with iron (sulfate)	10 mg/ml
Unicap M® plus iron (sulfate)	10 mg
Unicap Plus Iron® (sulfate)	18 mg

From Tong, T. G. and Banner, W., in *Current Emergency Therapy — 1984*, Edlich, R. F. and Spyker, D. A., Eds., Appleton-Century-Crofts, Norwalk, CT, 1984, 754. With permission.

child has ingested. Determination of the estimated dose taken is the first step in the triage of iron intoxication. In those ingestions in which the maximal amount ingested is less than 20 mg/kg of elemental iron, there is generally little risk for toxicity and no specific treatment is indicated. In the 20- to 60-mg/kg range, we intervene by advising the use of syrup of ipecac and follow-up these children at home by telephone. The intent here is to decontaminate and limit the contact time of the iron in the GI mucosa. A conservative estimate of more than 60 mg/kg of elemental iron should be an indication for medical evaluation. It should be kept in mind that these milligrams per kilogram amounts taken are intended only for use as guidelines in children younger than 5 years of age who have acutely ingested an unknown quantity of iron. Adolescent and adult overdoses should be viewed as intentional and require immediate medical referral regardless of the estimated dose taken. In addition, injury to the GI tract from large amounts of iron ingested by the older child or adult may occur, even though the total body burden of iron is not considered to be in the toxic range.

The clinical status of the patient is an important indicator of whether there is a need to initiate aggressive management in iron poisoning. In situations in which a patient becomes symptomatic, particularly with early GI symptoms, such as spontaneous emesis, epigastric

pain, and diarrhea, referral to a treatment facility is appropriate even if the estimated amount taken is low. In cases where lethargy, hypotonia, hypoperfusion, tachypnea, and tachycardia are seen, immediate chelation therapy is indicated regardless of the amount of iron reportedly ingested.

The serum iron level is another major determinant in the decision to institute aggressive therapy in cases of iron intoxication. The utility of serum iron determinations has been a source of controversy for many years. Critical to an understanding of the use of serum iron concentrations is an appreciation of the role of the total iron-binding capacity (TIBC). The iron-binding proteins in serum are regulated physiologically, but generally allow 200 to 300 μg/dl or iron-binding capacity. In theory, iron toxicity is more likely to occur when the serum iron concentration exceeds the ability of plasma proteins to bind it. In practice, however, the correlation between toxicity and iron-binding capacity appears to be relatively poor. The iron-binding capacity primarily measures a high-affinity binding site. Lower binding affinities may be present, as well as rapid transfer to iron-binding sites in liver and tissue that can remove free iron from the circulation. Occasionally, unusual results of TIBC may occur. Thus, the use of TIBC may occasionally be unreliable in severe iron intoxication.

It is also important to consider the impact of timing on the collection of serum iron concentrations. Because iron undergoes multicompartment kinetics with a fairly rapid distribution phase followed by an extremely long terminal elimination phase, dramatic chagnes in serum concentration may occur within the first few hours following ingestion. In addition, variable disintegration time of the tablets and capsules may also alter serum iron concentration-time relationships. Because of these factors, we have generally tired to measure serum iron concentrations between 4 and 6 h postingestion. During this time, the majority of oral iron preparations will have solubilized and serum iron will not yet have undergone complete distribution into tissue.

Iron concentrations in excess of 1000 μg/dl are associated with a dramatic increase in morbidity.[10] In addition, an unacceptable risk of shock is present (20%) when serum iron concentrations exceed 500 μg/dl. Thus, there is very little argument that a serum iron concentration greater than 500 μg/dl strongly supports the need for aggressive therapy.

Serum iron concentrations that fall between 300 and 500 μg/dl are often the greatest source of confusion. Our recommendations for patients with serum iron determinations in this range have been to begin chelation therapy if the iron concentration exceeds the TIBC (when available) or if the patient is experiencing any of the signs and symptoms of iron toxicity. The white blood cell count and serum glucose may be used as further indicators of systemic toxicity.[11] An abdominal radiograph should also be obtained in order to determine whether a large mass of tablets remains to be absorbed. Solid dosage forms of iron are sometimes evident on X-ray 6 to 8 h following their ingestion. As dissolution of tablets and capsules containing iron takes place, discrete forms may not be easily discerned; amorphorous densities may appear instead. Solutions and suspensions at high concentrations of iron, however, may be visualized.

It should be obvious that no single laboratory value or single historical or physical finding should be used as evidence to withhold therapy. Even when there are "normal" serum iron concentrations, it is reasonable to initiate chelation therapy in patients who are manifesting major clinical signs of toxicity. A negative X-ray examination does not alone rule out the possibility of severe poisoning from disintegrated iron tablets. By taking the approach of using the estimated total body burden of elemental iron, clinical signs and symptoms, and the serum iron concentration, a more rational approach to the initiation of chelation therapy can be undertaken.

B. DECONTAMINATION

The use of syrup of ipecac and gastric lavage in clinical toxicology situations is usually a relatively straightforward discussion. For iron intoxication, however, there is some con-

troversy and confusion on this issue. Based on our own case experiences, we offer the following recommendations:

1. Basic gastric decontamination procedures should be undertaken with as little delay as possible. The use of syrup of ipecac as an immediate measure in the home or during transportation to a treatment facility is a reasonable approach in the awake and alert child. It may be desirable to additionally lavage a child once the hospital or other medical setting has been reached, especially if the quantity of ingested iron is reportedly large. Any time delay in the initiation of decontamination procedures to await specific chelating or complexing agents is unacceptable.
2. The use of complexing or chelating agents is unnecessary in the initiation of gastric lavage, because it is primarily a mechanical procedure to remove substances from the stomach.
3. Complexation with phosphate solution should be avoided.
4. The use of conventional antacid preparations or 5% sodium bicarbonate may be useful in complexing iron and decreasing the corrosive effects of stomach acid upon a denuded gastric mucosa.
5. There are not data to support the use of intragastric deferoxamine, and we have chosen to avoid this approach.
6. Activated charcoal is not effective for complexing residual iron in the gastrointestinal tract.

Following gastric decontamination, abdominal radiography may be useful to identify large masses of iron still intact in the GI tract. The finding of multiple or amorphous densities in the stomach requires that additional attempts at removal be understaken. Only in rare circumstances in which a large number of densities are apparent and attempts at alternate GI decontamination have failed would surgical removal be indicated. If surgical intervention is contemplated, radiography immediately prior to operation is mandated to ensure that tablets or capsules have remained in one location.

1. Laboratory Evaluation

Laboratory evaluation in acute iron intoxication should be directed toward those tests that will aid in the diagnosis and delivering of supportive care. Once the initial serum iron has been determined to be in the toxic range, there is little value to continued determinations of the serum iron or TIBC. In many institutions, the TIBC cannot be determined on an immediate basis; therefore, it becomes of relatively little value in monitoring therapy. In cases of severe GI toxicity due to iron, it is wise to obtain a hematocrit and cross-match for blood products if hemorrhage occurs. Additional baseline laboratory tests may include a white blood cell count and serum glucose determination to assess the level of acuity of the patient. An arterial blood gas or bicarbonate determination is important in monitoring the development of metabolic acidosis. Serum calcium and coagulation studies are also useful in severe intoxications; these may become abnormal. Determination of serum electrolytes is useful in the overall fluid management of the patient. There is little value in obtaining indicators of hepatic function early in the course of iron intoxication; however, it is reasonable to obtain transaminase determinations after the first 24 h to assess whether or not hepatic damage has occurred.

C. CHELATION THERAPY

Deferoxamine should be initiated intravenously by continuous infusion in all cases of serious iron poisoning. We recommend a slow continuous intravenous infusion of deferoxamine (deferoxamine should never be given rapidly), starting with a dose of 15 mg/kg/

h. Some authors recommend decreasing the rate to 6 mg/kg/h after the first 4 to 5 h of chelation therapy. Chelation therapy at 15 mg/kg/h should be continued while evidence of the iron-deferoxamine complex in the urine is seen.[12] A major exception to the use of intravenous deferoxamine is when prolonged interhospital transport time is involved. If close monitoring of intravenous infusions is not possible, it may be more judicious to give deferoxamine by the intramuscular route. Intramuscular administration of deferoxamine is painful and often responsible for local reactions at the injection site. Adverse effects of deferoxamine therapy include hypotension and tachycardia following too-rapid infusion; urticaria and erythema have also been noted.

The decision to discontinue chelation therapy should be made on the basis of several parameters. Traditionally, a change in urine color from normal to "vin rose" has been interpreted as an indication for continued chelation therapy. Observation in which a high concentration of chelated iron was present in urine without changes in color has been reported.[13] Thus, in a situation in which the serum iron concentration is well above 500 μg/dl and no change in urine color is observed, it is reasonable to continue chelation therapy until the serum iron has fallen to less than 300 μg/dl. In those patients with serum iron concentrations in the 300 to 500 μg/dl range and even when the TIBC is exceeded, frequently no change in urine color will occur during chelation. In most patients in whom the traditional "vin rose" color does occur, it is suggested that chelation therapy continue until a normal urine output of normally colored urine occurs. When the urine color has cleared, continuing chelation for an additional 24 h or stopping it immediatley have both been suggested. This decision should be based on the assessment of severity.

D. SUPPORTIVE CARE

Supportive care is the singular and most important mode of management of the iron-intoxicated patient. Initial attempts should be made to minimize the impact of iron tablets on the GI tract and to monitor for the presence of GI hemorrhage. With the fluid loss in the GI tract and the generalized loss of capillary integrity in the body, the most important factor in supportive care is to sustain the intravascular volume. Elevation of the hematocrit in iron intoxication is an important indicator of fluid volume loss and should be dealt with aggressively. Monitoring of urine output, heart rate, and assessment of tissue perfusion along with measurement of the central venous pressure should guide fluid therapy. Large fluid volumes may be needed during the first 24 h of management in order to avoid the risks of hypovolemic shock. In addition, correction of acidosis and hypocalcemia and monitoring of serum electrolytes are important in the management of iron intoxication.

E. OTHER RESOURCES

Chelation of iron with deferoxamine is a relatively ineffective way of binding large amounts of iron. The amount of iron removed from the body during chelation is a relatively small percentage of the total body burden of iron. Recommendation that deferoxamine be given until either a maximum 24-h dose of 6 g or 240 mg/kg is reached is not supported well with current data. As 1 g of deferoxamine chelates only 85 mg elemental iron, this maximum dose recommendation may be insufficient to neutralize the iron taken in large overdoses. When serum iron concentrations are markedly elevated, some have suggested the use of exchange transfusion.[14] From a toxicokinetic standpoint, if iron has remained in the intravascular compartment as evidenced by serum concentrations greater than 5000 μg/dl, there may be some advantages to exchange transfusion under these rare circumstances. There are no data to suggest that the routine management of acute iron intoxication requires this kind of intervention. Hemodialysis may effectively remove the complex of deferoxamine and iron, but is considered to be a less efficient method for removal than is glomerular filtration. Hemodialysis is rarely suggested unless renal failure has occurred. The vast

majority of iron intoxications will respond to standard intravenous chelation plus good supportive measures and will unlikely require more aggressive therapy.

IV. CONTROVERSIES IN THE MANAGEMENT OF IRON INTOXICATION

As with many toxicologic issues, the inability to perform well-controlled prospective studies has led to a great deal of confusion and controversy in the management of the iron-intoxicated patient. Conflicting suggestions and lack of consensus on management provide a continuing source of difficulty for the clinician. Recognizing that this area is controversial, the clinician must use his or her judgment in deciding the most rational course for an individual patient. It is important to recognize that some procedures such as gastrotomy, although not useful for the vast majority of patients, might be beneficial in a minority of patients. The role of the clinician remains to balance the risks and benefits for his or her patient.

A. INTRAGASTRIC COMPLEXATION

The use of intragastric complexation in iron intoxication is one ongoing controversy. Sodium bicarbonate and phosphate solution have been suggested to promote the formation of insoluble iron complexes.[11,15] Studies have demonstrated that phosphate can decrease iron absorption in normal human subjects, but fail to address the same issue in iron intoxication. Sodium bicarbonate and sodium phosphate will form relatively water-insoluble iron complexes in the stomach when given orally.[16] These complexes are labile, and a subsequent decrease in pH into the normal range of gastric fluid can allow iron to again become soluble.

There seems to be little advantage in using either of these solutions during the mechanical lavage of the stomach. When normal saline is introduced into the stomach, it is hardly relevant whether the material to be removed by lavage is in a soluble or insoluble form. The more important question is whether the residual iron that is not effectively removed by lavage or emesis can be maintained in an insoluble state for a prolonged period of time, thus making it less absorbable. Sodium bicarbonate administered in sufficient quantities to maintain the GI environment in the desired pH range would require repeated doses, which may result in rises in serum sodium. In the case of phosphate-containing solutions, the amount of phosphate absorbed from the use of this as a lavage solution during iron intoxication has been shown to be hazardous.[17,18] Hypocalcemia, hyperphosphatemia, severe acidosis, and hypernatremia have occurred as a consequence of repeated and indiscriminate use of phosphate solutions. Several clinicians have advocated the use of magnesium hydroxide or Milk of Magnesia® to decrease the solubility of iron.[19] Clinical trials on the effectiveness of magnesium hydroxide in humans are still needed. With an iron-damaged stomach, it seems reasonable to protect the stomach and upper duodenum from further damage from stomach acids. To this end, the administration of conventional antacid preparations with low bioavailability for their buffering ions seems to present a useful and safe alterntive to the previously recommended therapies. To avoid iatrogenic complications, however, it should be remembered that magnesium-containing antacids may lead to increases in serum magnesium if renal function is impaired.

The use of deferoxamine as an intragastric chelating agent has received a great deal of discussion in the literature. The relevant considerations in intragastric chelation of iron with deferoxamine include:

1. Does complexation decrease the absorption of iron?
2. Does intragastric complexation decrease the toxicity of iron following absorption?
3. Is the intragastric administration of deferoxamine associated with any adverse effects?

Unfortunately, very few studies have been designed to directly address these questions.

Studies addressing the question of bioavailability of the iron-deferoxamine complex have failed to provide useful information for the management of iron intoxication. When deferoxamine is used orally in chronic iron overload states, the bioavailability of iron is decreased.[20,21] However, in animal studies of acute iron overdose, the iron-deferoxamine complex is absorbed.[22]

The question whether iron absorbed as the iron-deferoxamine complex is less toxic than iron absorbed alone and subsequently chelated with intravenous deferoxamine is more difficult to answer. It has been postulated that iron when absorbed as the complex may be less toxic because of its lower volume of distribution.[23] Under these conditions, distribution into cells and thus effects on the mitochondria could be avoided.

The potential adverse effect of intragastric deferoxamine is that it might somehow increase the rate of dissolution or increase the rate of absorption associated with acute iron ingestions. Evidence for this possibility comes from studies which show the biochemical parameters of iron toxicity associated with combined therapy of intravenous and oral deferoxamine appeared to progress more rapidly than those associated with intravenous deferoxamine alone.[22] Other investigators have found that concentrations of iron in the liver were increased in the presence of deferoxamine in the GI tract.[24]

As in the case of sodium bicarbonate, it seems irrelevant which fluid is used for mechanically washing out the stomach. Thus, it would appear to be an expensive approach to use deferoxamine merely as a lavage fluid. In addition to cost, only a limited supply of deferoxamine is usually available to the clinician in many rural practice settings.

B. ADMINISTRATION OF DEFEROXAMINE

The administration of deferoxamine as an intramuscular preparation is open to much discussion. Intramuscular or subcutaneous use of deferoxamine has been advocated for patients with chronic iron overload, such as those receiving repeated blood transfusions. Adverse effects noted with the use of deferoxamine by either the intramuscular or intravenous route are few.[25] The most stiking difference between intravenous and intramuscular deferoxamine is the effect of a rapid bolus of intravenous deferoxamine on blood pressure. This acute hypotension produced is most likely caused by the release of endogenous histamine.[26]

In cases of severe iron intoxication, it is difficult to recommend intramuscular medications when poor tissue perfusion is a primary complication early in the intoxication. Deferoxamine used as a continuous intravenous infusion at 15 mg/kg/h has been demonstrated to be safe and not associated with hypotension. One difficulty with current recommendations for the use of intravenous deferoxamine is the lack of agreement on maximal infusion rates.[12] Some have suggested initiating therapy with an infusion of 15 mg/kg/h and then decreasing this rate of infusion to 6 mg/kg/h after stabilization. The intravenous access can also be used conveniently to provide volume expansion should it be necessary as is often the case in severe iron intoxication.[27]

Deferoxamine administration in iron overdose may be accompanied by an increased risk for developing *Yersinia enterocolitica* septicemia. Case reports of this unusual infection postulate that deferoxamine, a siderophore, is acting as a growth factor *in vitro*, enhancing the virulence of the pathogen.[28]

C. GASTROTOMY FOR TABLET REMOVAL

Two recent reports have described the surgical removal of iron tablets from the GI tract following severe iron intoxication.[29,30] The indication for surgery used in both of these circumstances was the abdominal radiograph. When a large number of opacities are identified in a single location, it may be reasonable to consider surgical intervention. No real estimate of the risk-benefit relationship for this approach is known. The clinician must consider the stability of the patient, the number of tablets seen on radiograph, and the potential for other

procedures such as lavage, emesis, and catharsis to successfuly remove these tablets. The propensity from some iron tablet preparations to form concretions may mandate this approach. Surgical intervention should be considered only if a large number of tablets inaccessible to emesis, lavage, or gastroscopy appear to be present, and the clinician should obtain radiographs immediately prior to surgical intervention. Laparotomy to remove iron concretions should not be considered a routine measure in iron intoxication.

D. HEMODIALYSIS AND EXCHANGE TRANSFUSION

As with many forms of intoxication, hemodialysis has been investigated in iron intoxication. It does appear that the complex of deferoxamine and iron can be removed by dialysis.[31] When confronted with the possibility of renal failure, this seems a reasonable approach; however, there appears to be relatively little advantage to the initiation of hemodialysis in cases in which renal function is normal.

Exchange transfusion is another area of controversy for many intoxicated states. Currently, recommended doses of deferoxamine will chelate very little of the total body burden of iron in a serious overdose. Exchange transfusion has been demonstrated to increase the rate of iron removal by as much as 30-fold when compared with chelation therapy alone in animal studies.[14] This approach should only be seriously considered in situations where other conventional therapies have failed and where serum iron concentrations are extremely high. Given the poor affinity of iron for charcoal, the use of charcoal hemoperfusion would offer little benefit, if any. Since the iron deferoxamine complex does bind to charcoal, further study and experience may suggest that this is a possible therapeutic approach.[30]

V. SUMMARY

Iron poisoning is a major toxicologic problem. Failure to recognize the severity of iron intoxication may result in an inappropriate level of intervention. Estimates of the total body burden of iron, clinical symptoms, and the serum concentration are needed before an appropriate decision can be made to initiate therapy. In severe intoxication, the use of intravenous deferoxamine is indicated, along with supportive care and particular attention given to maintaining the intravascular volume. Other measures should include correction of metabolic acidosis and disorders of coagulation. Replacement of blood components is necessary when there is evidence of GI hemorrhage. Measures such as hemodialysis and exchange transfusion should be reserved for those unusual poisonings in which more conventional therapy has been unsuccessful. In rare cases of iron intoxication, late sequelae such as hepatic necrosis and GI scarring with obstruction may occur. The prompt recognition and initiation of management of children with acute iron poisoning is the single most critical element in decreasing the morbidity and mortality associated with these products.

REFERENCES

1. **Litovitz, T. L., Matyunas, N., Schmitz, B., and Martin, T. G.,** 1987 Annual Report of the American Association of Poison Control Centers National Data Collection System, *Am. J. Emer. Med.*, 6, 479, 1988.
2. **Krenzelok, E. P. and Hoff, J. V.,** Accidental childhood iron poisoning: a problem of marketing and labeling, *Pediatrics*, 63, 591, 1979.
3. **Covey, T. J.,** Ferrous sulfate poisoning, *J. Pediatr.*, 64, 218, 1954.
4. **Whitten, C. F., Chen, Y. C., and Gibson, G. W.,** Studies in acute iron poisoning. III. The hemodynamic alterations in acute experimental iron poisoning, *Pediatr. Res.*, 2, 479, 1968.
5. **Reissmann, K. R., Coleman, T. J., Budal, B. A., and Moriarty, L. R.,** Acute intestinal iron intoxication. I. Iron absorption, serum iron, and autopsy findings, *Blood*, 10, 35, 1955.

6. **Reissmann, K. R. and Coleman, T. J.**, Acute intestinal iron intoxication. II. Metabolic, respiratory, and circulatory effects of absorbed iron salts, *Blood*, 10, 46, 1955.
7. **Rosenmund, A., Haeberli, A., and Straub, P. W.**, Blood coagulation and acute iron toxicity, *J. Lab. Clin. Med.*, 103, 524, 1984.
8. **Wilson, S. J., Heath, H. E., Nelson, P. L., and Ens, G. G.**, Blood coagulation in acute iron intoxication, *Blood*, 13, 483, 1958.
9. **Whitten, C. F. and Brough, A. J.**, The pathophysiology of acute iron poisoning, *Clin. Toxicol.*, 4, 585, 1971.
10. **Westlin, W. F.**, Deferoxamine as a chelating agent, *Clin. Toxicol.*, 4, 597, 602, 1971.
11. **Lacouture, P. G., Wason, S., Temple, A. R., Wallace, D. K., and Lovejoy, F. H., Jr.**, Emergency assessment of severity in iron overdose by clinical and laboratory methods, *J. Pediatr.*, 99, 89, 1981.
12. **Peck, M. G., Rogers, J. F., and Rivenbark, J. F.**, Use of high doses of deferoxamine in an adult patient with acute iron overdosage, *J. Toxicol. Clin. Toxicol.*, 19, 865, 1982.
13. **Freeman, D. A. and Manoguerra, A. S.**, Absence of urinary color change in a severely iron-poisoned child treated with deferoxamine, *Vet. Hum. Toxicol.*, 23, 351, 1981.
14. **Movassaghi, N., Purugganan, G. G., and Lekin, S.**, Comparison of exchange transfusion and deferoxamine in the treatment of acute iron poisoning, *J. Pediatr.*, 75, 604, 1969.
15. **Benjamin, B. I., Cortell, S., and Conrad, M. E.**, Bicarbonate-induced iron complexes and iron absorption, *Gastroenterology*, 53, 389, 1967.
16. **Czajka, P. A., Konrad, J. D., and Duffy, J. P.**, Brief clinical and laboratory observations, *J. Pediatr.*, 98, 491, 1981.
17. **Bachrach, L., Correa, A., Levin, R., and Grossman, M.**, Iron poisoning: complications of hypertonic phosphate lavage therapy, *J. Pediatr.*, 94, 147, 1979.
18. **Geffner, M. E. and Opas, L. M.**, Phosphate poisoning complicating treatment for iron ingestion, *Am. J. Dis. Child.*, 134, 509, 1980.
19. **Chadwick, E. W. and Corby, D. G.**, Is milk of magnesia a potentially effective antidote for acute iron overdose?, *Vet. Hum. Toxicol.*, 24, 298, 1982.
20. **Hwang, and Brown, E. B.**, Effect of desferrioxamine on iron absorption, *Lancet*, 50, 135, 1965.
21. **Jacobs, A., Kaye, D., and Trevett, D.**, The chelation of iron during intestinal absorption, *J. Lab. Clin. Med.*, 74, 212, 1969.
22. **Whitten, C. F., Gibson, G. W., Good, M. H., Goodwin, J. F., and Brough, A. J.**, Studies in acute iron poisoning. I. Desferrioxamine in the treatment of acute iron poisoning: clinical observations, experimental studies, and theoretical considerations, *Pediatrics*, 36, 322, 1965.
23. **Robotham, J. L. and Lietman, P. S.**, Acute iron poisoning, *Am. J. Dis. Child.*, 134, 875, 1980.
24. **Brunner, H., Hedwall, H. K., and Schmid, K.**, Beeinfulssung einer akuten, enteraten ie-II-vergiftung am hund durch desferrioxamine, *Helv. Physiol. Pharmacol. Acta*, 23, C81, 1965.
25. **Batey, R., Scott, J., Jain, S., and Sherlock, S.**, Acute renal insufficiency occurring during intravenous desferrioxamine therapy, *Scand. J. Haematol.*, 22, 277, 1979.
26. **Butterworth, E. E. and Scott, C. W.**, Iron mobilizing and histamine releasing properties of desferrioxamine, *Clin. Res.*, 13, 33, 1965.
27. **Henretig, F. M., Kari, S. R., and Weintraub, W. H.**, Severe iron poisoning treated with enteral and intravenous deferoxamine, *Ann. Emerg. Med.*, 12, 306, 1983.
28. **Mofenson, H. C., Caraccio, T. R., and Charleff, N.**, Iron sepsis: *Yersinia enterocolitica* septicemia possibly caused by an overdose of iron (letter), *N. Engl. J. Med.*, 316, 1092, 1987.
29. **Venturelli, J., Kwee, Y., Morris, N., and Cameron, G.**, Gastrotomy in the management of acute iron poisoning, *J. Pediatr.*, 100, 768, 1982.
30. **Peterson, C. D. and Fifield, G. C.**, Emergency gastrotomy for acute iron poisoning, *Ann. Emerg. Med.*, 9, 33, 1980.
31. **Richardson, J. R., Sugerman, D. L., and Hulet, W. H.**, Extraction of iron by chelation with desferrioxamine and hemodialysis, *Clin. Res.*, 15, 368, 1967.
32. **Yonker, J., Banner, W., and Picchioni, A.**, Absoption characteristics of iron and deferoxamine onto charcoal, *Vet. Hum. Toxicol.*, 22 (Suppl. 2), 75, 1980.
33. Arizona Poison Control System, Unpublished data.

Appendix C

Human & Experimental TOXICOLOGY

Volume 20 Number 7 July 2001

www.hetjournal.com

Human & Experimental Toxicology

Human & Experimental Toxicology (0960-3271) is published monthly by Arnold, part of the Hodder Headline Group, 338 Euston Road, London NW1 3BH, UK. The known office of publication is The Sheridan Press, 450 Fame Avenue, Hanover, PA 17331, USA. Periodicals postage is pending at Hanover, PA. Postmaster – please send address changes to Turpin North America, 440 Creamery Way, Suite A, Exton, PA 19341, USA. *Human & Experimental Toxicology* is affiliated to the French Society of Toxicology.

Scope The Editors will consider papers on all aspects of experimental and clinical studies of functional, biochemical and structural disorders. Attention is paid to experimental work and studies of basic toxicological and biological mechanisms and to clinical reports of important cases.

Human & Experimental Toxicology is indexed by Current Contents, Index Medicus, Excerpta Medica, BIOSIS and CABS.

Editorial Manuscripts (original plus 2 copies) and all editorial correspondence should be sent to:

Robert Kroes
Editor-in-Chief
Human & Experimental Toxicology
c/o P.O. Box 114
NL-3760 AC SOEST
The Netherlands

Courier shipments only
Seminariehof 38
NL 3768 EE SOEST
The Netherlands
Tel: +31 35 642 2393. Fax: +31 35 622 3442
Email: rmkroes@worldonline.nl

Editor for the Americas
A. Wallace Hayes
The Gillette Company
Prudential Tower Building
Boston, Massachusetts
02199
USA
Tel: +1 617 421 7380 Fax: +1 617 421 7360
Email: awallace_hayes@gillette.com

Publisher All business correspondence, advertisement enquiries, supplement enquiries, reprint orders and permission requests should be addressed to *Human & Experimental Toxicology*, Arnold Journals, 338 Euston Road, London NW1 3BH, UK. Tel: +44 20 7873 6339, Fax: +44 20 7873 6325, E-mail: arnoldjournals@hodder.co.uk

WWW *Human & Experimental Toxicology's* homepage can be found at www.hetjournal.com. Editor's details, scope, subscription prices, reprint ordering, sample copy ordering, supplement information and contact names for the publishing, production, marketing and advertising departments are available for all Arnold journals.

Subscriptions

2001 Subscription rates

INSTITUTIONAL SUBSCRIPTIONS
(print and electronic online)

US	$726
Europe	£506
Rest of the World	£506

Individual subscriptions (print only)

US	$436
Europe	£304
Rest of the World	£304

Orders and payment

Turpin Distribution Services Limited handle the distribution of this journal. Orders accompanied with payment (cheques made payable to Turpin Distribution Services Limited) should be sent directly to Turpin Distribution Services Limited, Blackhorse Road, Letchworth, Hertfordshire SG6 1HN, UK Tel: +44 (0) 1462 672 555. Fax: +44 (0) 1462 480 947

Customers should make payments by cheque in sterling payable on a UK clearing bank or in US dollars payable on a US clearing bank.

Human & Experimental Toxicology (2001) 20, 365–372

www.arnoldpublishers.com/journals

Deferoxamine toxicity in hepatoma and primary rat cortical brain cultures

DW Christensen*,[1], R Kisling[1], J Thompson[2] and MA Kirby[1]

[1]*Department of Pediatrics, Loma Linda University School of Medicine, Loma Linda, California 92350, USA;*
[2]*Department of Biology, California State University, San Bernardino, California 92407, USA*

Deferoxamine is commonly used for treatment of iron intoxication. Because the usual dose is unable to chelate sufficient iron before severe injury occurs, "high-dose" deferoxamine treatment has been proposed. However, several authors have reported severe toxicity after deferoxamine therapy. Although the hemodynamic effects are well described, the cellular toxicity of deferoxamine is unknown. Accordingly, we investigated the cellular toxicity of deferoxamine using *in vitro* techniques in two cell lines. Brain cells were harvested from fetal rats and cultured for 14–21 days before deferoxamine exposure. Using similar techniques, rat hepatoma cells were grown until confluent. Deferoxamine was added to the cultures to achieve final concentrations of 200–800 μg/ml, corresponding to *in vivo* infusion rates of 15–60 mg/kg/h. Deferoxamine was removed after 3 or 6 days by changing the medium. Subtoxic $FeCl_3$ (500 mg/dl) was concurrently added to identical cultures to determine if deferoxamine potentiated iron toxicity. Cell viability was measured by a colorimetric assay. The addition of deferoxamine (0.2, 0.4, 0.8 mg/ml) significantly decreased cell viability in both cell groups. The effect of deferoxamine on primary cortical brain cultures was similar for the three concentrations used, and was similar when examined either 72 h or 6 days later. In contrast, hepatoma cell cultures evidenced a dose-dependent cell loss that increased with the length of exposure. The addition of subtoxic amounts of $FeCl_3$ (500 μg/dl) in the presence of deferoxamine was protective in all cultures, and abolished deferoxamine-induced cell loss. Interestingly, the addition of serum albumin significantly reduced the amount of iron present in cells, suggesting its potential use to treat iron toxicity. These results suggest that deferoxamine, in the absence of iron, is toxic to cortical brain and hepatoma cells *in vitro*. *Human & Experimental Toxicology* (2001) 20, 365–372.

Keywords: Iron toxicity; deferoxamine; MTT; primary cortical cells; hepatoma cells; neurons; $FeCl_3$

Introduction

Iron intoxication is a common cause of morbidity and mortality among children. Lethal doses of iron, typically in the form of enticing, brightly colored vitamins, are readily available to, and frequently ingested by children. The pathophysiology of iron toxicology is complex and, at present, only partially understood. The severity of iron intoxication is directly related to the amount of elemental iron absorbed.[1] Serum iron levels peak between 2 and 6 h after iron ingestion,[2] and decrease to minimally elevated levels within 6[2] to 12[3] h after ingestion in humans. Within 6 h of ingestion, iron is transported to the mitochondria where it interrupts intermediary metabolism and disrupts cellular function. This typically results in multi-organ failure, pyloric or antral stenosis, hepatic cirrhosis and central nervous system dysfunction.[4] Currently, available therapy for severe iron intoxication includes decontamination therapy (such as gastric lavage and whole bowel irrigation) to prevent absorption, followed by administration of deferoxamine to chelate absorbed iron.[2]

Because medical therapy often is not sought until several hours after potentially lethal iron ingestion, toxic amounts of iron have frequently been absorbed before patients seek medical care. Hence, deferoxamine chelation therapy and supportive care are frequently the only available course of treatment for severe iron intoxication. The efficacy of chelation therapy is limited by deferoxamine's iron-binding capacity and its own cellular toxicity. The maximum recommended deferoxamine infusion rate is 15 mg/kg/h because larger doses may result in hypotension, as well as tachycardia and gastrointestinal complications (package insert). However, because iron binds deferoxamine in a 1:1 molar ratio, this dose only chelates up to 50% of a potentially lethal dose of

*Correspondence: DW Christensen, MD, Pediatric Critical Care, St. Luke's Regional Medical Center, 190 East Bannock Street, Boise, Idaho 83712-9987, USA
Received 1 September 2000; revised 1 February 2001; accepted 7 March 2001

iron (60 mg/kg) in the first 24 h of therapy. Because chelation therapy may be less effective once iron has entered the extracellular space, clinical practice has involved the use of high dosages of deferoxamine (up to 60 mg/kg/h) with aggressive fluid administration to overcome deferoxamine-induced hypotension. However, recent reports suggest that deferoxamine may have direct cellular toxicity.[5–9] Cellular toxicity of high-dose deferoxamine treatment has been associated with vision and hearing loss, adult respiratory distress syndrome (ARDS) and multi-organ failure.[7] At present, the relationship between deferoxamine and cellular toxicity is unclear and must be clarified before high-dose deferoxamine therapies can be safely used. Accordingly, the present study was designed to evaluate high-dose deferoxamine toxicity on two cell lines, hepatoma and primary cortical brain cells, using *in vitro* techniques.

Methods

Animals were cared for in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Two cell lines, primary cortical brain cells and hepatoma cells, were used. These cells represent two of the major organs affected by iron or deferoxamine toxicity and have different and unique characteristics. Primary cortical brain cell cultures are composed of several cell classes of neurons and glia and are principally post-mitotic following 2–3 weeks of post-platting growth. In contrast, hepatoma cells are homogeneous and mitotically active under a variety of culture conditions. The use of these two cells types was important for distinguishing cellular toxicity on a stable cell population with limited capability to replace cells from a mitotically active cell population that is continuously adding new cells. Additionally, the *in vitro* technique used in this study facilitated determination of deferoxamine toxicity without the confounding interaction of other organ effects such as hypotension, pulmonary edema or organ failure. Moreover, it permitted quantification of toxic levels for specific tissues that are problematic in whole animal studies. The data derived from this initial study will be used to guide future whole animal studies.

Cell culture

Primary cortical brain cultures

To obtain primary cortical brain cultures, pregnant Sprague–Dawley rats at 17 days of gestation were placed into a CO_2-filled chamber until unconscious, after which cervical dislocation euthanasia was performed. Fetuses were removed by rapid Cesarean section; the brains removed and placed into cold minimum essential medium (MEM) supplemented with D-glucose and 10% fetal bovine serum (FBS). After cooling, the cerebrums were placed into fresh medium containing trypsin for 10 min. The trypsin was then neutralized by the addition of 5% serum and cells dissociated in additional medium by gentle trituration. Cells were diluted to a concentration of 3–3.5 million cells/ml, and 300,000–350,000 cells were plated per well onto 96-well plates (100 μl medium/well). Cells were cultured at 37°C in 5% CO_2, 95% air for 14–21 days before deferoxamine exposure.

Hepatoma culture

Rat hepatoma cells were cultured in MEM supplemented with D-glucose and 15% FBS serum in standard tissue culture flasks. Cells were harvested 3 days before deferoxamine exposure using 0.6% trypsin solution, and triturated to dissociate cells. After centrifugation at 2000 rpm for 3 min, the medium was removed. Cells were diluted in medium to achieve 500,000 cells/ml, and 100 μl (50,000 cells) per well was plated onto 96-well plates and allowed to grow to confluence. The medium was replaced every 3 days in both the primary cortical brain and the hepatoma cultures.

Deferoxamine exposure

Seventy-two-hour primary brain and hepatoma culture exposure

Primary brain and hepatoma cultures were incubated in different concentrations of deferoxamine for 72 h. In both primary brain and hepatoma cultures, the culture medium was replaced with 100 μl fresh, indicator-free medium immediately prior to deferoxamine exposure. Deferoxamine was dissolved in culture medium to form concentrated solutions (0, 2.4, 4.8 and 9.6 mg/ml). Next, 10 μl of the deferoxamine solution was added to the medium in each well to achieve final concentrations of 0 (control), 200, 400 and 800 μg/ml. These concentrations were chosen to correspond to estimated serum levels for *in vivo* deferoxamine infusion rates of 0, 15, 30 and 60 mg/kg/h (calculated using V_d of 0.6 l/kg). Cultures were returned to the incubator for 72 h, after which viability assays were performed. The time of exposure (72 h) was chosen to approximate clinical exposure for deferoxamine treatment. All time points described refer to the interval from the beginning of deferoxamine exposure until the referenced time.

Six-day trials

One of the central questions in this study was the long-term effect of deferoxamine exposure. Accordingly, additional groups of primary brain and hepatoma cultures were exposed to deferoxamine for 6 days. Culture medium was changed immediately before deferoxamine exposure and 10 μl of concentrated deferoxamine solution added to the medium to achieve 0 (control), 200, 400 or 800 μg/ml final concentration. In one group of cultures, deferoxamine was removed after 3 days by changing to fresh culture medium and cultures were returned to the incubator for an additional 3 days. This group provided information on the ability of the cells to recover from deferoxamine toxicity, or alternatively, of delayed toxic effects. A second group of cultures was incubated in deferoxamine-containing medium for 6 days. The second group was important for examination of continuous, long-term exposure to deferoxamine. Cell viability assays were performed in both groups at 6 days and compared to untreated matched controls.

Concurrent iron exposure

Previous reports have associated excessive chelation with deferoxamine toxicity. To determine if iron affected deferoxamine toxicity in our cultures, $FeCl_3$ was added to achieve 500 μg/ml final concentration with deferoxamine in identical cultures described above. $FeCl_3$, 0.5 mg/ml, was dissolved in Hank's balanced salt solution (HBSS), and 10 μl was added to each well. This level of iron minimally affects cell viability and represents a subtoxic dose. To maintain equal volumes of medium within wells, 10 μl of HBSS was added to all wells not treated with iron.

Assessment of toxicity

Cell viability was measured by colorimetric assay using the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).[10] Tetrazolium salts such as MTT can be used to measure the activity of dehydrogenase enzymes, which are present only in living cells. These enzymes primarily in active mitochondria cleave the tetrazolium ring. Cleavage of the tetrazolium ring results in formation of a dark blue formazan product in proportion to the number of live cells present.

The MTT assay was performed by adding 20 μl of 2.5 mg MTT/ml solution to each culture well, and incubating for 5 h at 37°C. SDS (100 μl, 10%) was then added and allowed to dissolve the formazan crystals overnight. After assuring that all cells were lysed and all crystals were dissolved, optical density of each well was read at 620 nm and recorded. Neither deferoxamine nor $FeCl_3$ had any effect on the MTT assay as measured in control wells and wells filled with dH_2O.

Statistical analysis

Each data point reported in this study consists of the average of eight or more wells. Mean values ($\pm$SEM) of four sets of animals and eight hepatoma cultures were calculated (Figures 1–5). The mean values were compared using an overall multivariate ANOVA with repeated measures to determine main effects, followed by one-way ANOVA with Duncan's post-hoc testing for individual comparisons.

Results

Deferoxamine exposure produced a reduction of cell viability in both cortical brain and hepatoma cultures. Concurrent exposure to 500 μg/dl of $FeCl_3$ and deferoxamine was protective in all cultures.

Primary brain culture

Cortical brain cell viability is shown in Figure 1, grouped by deferoxamine exposure and measurement time. In the absence of deferoxamine exposure (0 Deferox), baseline brain cell viability slightly decreased over time as evidenced by the decreased

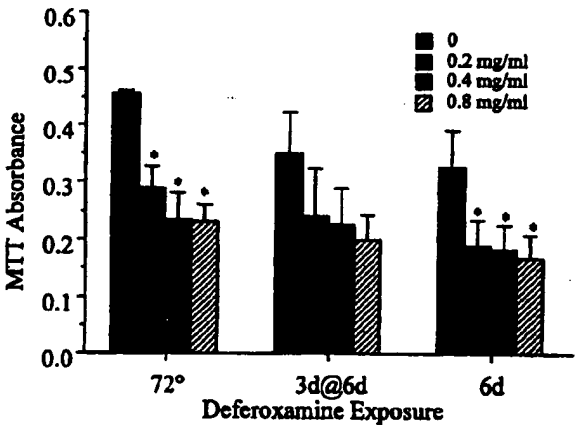


Figure 1 Deferoxamine toxicity in primary neuronal cell cultures as measured by MTT absorbance. Deferoxamine was added to primary cortical brain cultures at 0 (control), 0.2, 0.4 and 0.6 mg/ml. The cultures were divided into three groups: a 72-h exposure group (72°), a 3-day deferoxamine exposure group examined on day 6 (3d@6d), and a 6-day exposure group (6d). The addition of deferoxamine decreased cell viability at all three concentrations used (*$P<0.01$). Moreover, the effect was similar across the three concentrations used. The addition of deferoxamine significantly reduced cell viability in the 72-h (72°) and 6-day (6d) groups, and resulted in a trend of reduced viability in cell groups exposed for 3 days but allowed to recover until day 6 (3d@6d)

Six-day trials

One of the central questions in this study was the long-term effect of deferoxamine exposure. Accordingly, additional groups of primary brain and hepatoma cultures were exposed to deferoxamine for 6 days. Culture medium was changed immediately before deferoxamine exposure and 10 μl of concentrated deferoxamine solution added to the medium to achieve 0 (control), 200, 400 or 800 μg/ml final concentration. In one group of cultures, deferoxamine was removed after 3 days by changing to fresh culture medium and cultures were returned to the incubator for an additional 3 days. This group provided information on the ability of the cells to recover from deferoxamine toxicity, or alternatively, of delayed toxic effects. A second group of cultures was incubated in deferoxamine-containing medium for 6 days. The second group was important for examination of continuous, long-term exposure to deferoxamine. Cell viability assays were performed in both groups at 6 days and compared to untreated matched controls.

Concurrent iron exposure

Previous reports have associated excessive chelation with deferoxamine toxicity. To determine if iron affected deferoxamine toxicity in our cultures, $FeCl_3$ was added to achieve 500 μg/ml final concentration with deferoxamine in identical cultures described above. $FeCl_3$, 0.5 mg/ml, was dissolved in Hank's balanced salt solution (HBSS), and 10 μl was added to each well. This level of iron minimally affects cell viability and represents a subtoxic dose. To maintain equal volumes of medium within wells, 10 μl of HBSS was added to all wells not treated with iron.

Assessment of toxicity

Cell viability was measured by colorimetric assay using the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).[10] Tetrazolium salts such as MTT can be used to measure the activity of dehydrogenase enzymes, which are present only in living cells. These enzymes primarily in active mitochondria cleave the tetrazolium ring. Cleavage of the tetrazolium ring results in formation of a dark blue formazan product in proportion to the number of live cells present.

The MTT assay was performed by adding 20 μl of 2.5 mg MTT/ml solution to each culture well, and incubating for 5 h at 37°C. SDS (100 μl, 10%) was then added and allowed to dissolve the formazan crystals overnight. After assuring that all cells were lysed and all crystals were dissolved, optical density of each well was read at 620 nm and recorded. Neither deferoxamine nor $FeCl_3$ had any effect on the MTT assay as measured in control wells and wells filled with dH_20.

Statistical analysis

Each data point reported in this study consists of the average of eight or more wells. Mean values ($\pm$SEM) of four sets of animals and eight hepatoma cultures were calculated (Figures 1–5). The mean values were compared using an overall multivariate ANOVA with repeated measures to determine main effects, followed by one-way ANOVA with Duncan's post-hoc testing for individual comparisons.

Results

Deferoxamine exposure produced a reduction of cell viability in both cortical brain and hepatoma cultures. Concurrent exposure to 500 μg/dl of $FeCl_3$ and deferoxamine was protective in all cultures.

Primary brain culture

Cortical brain cell viability is shown in Figure 1, grouped by deferoxamine exposure and measurement time. In the absence of deferoxamine exposure (0 Deferox), baseline brain cell viability slightly decreased over time as evidenced by the decreased

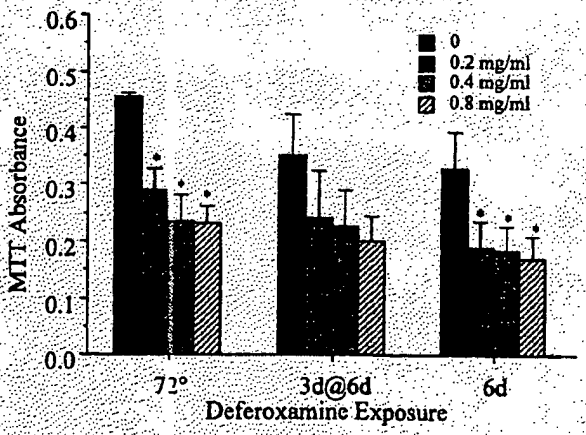


Figure 1 Deferoxamine toxicity in primary neuronal cell cultures as measured by MTT absorbance. Deferoxamine was added to primary cortical brain cultures at 0 (control), 0.2, 0.4 and 0.6 mg/ml. The cultures were divided into three groups: a 72-h exposure group (72°), a 3-day deferoxamine exposure group examined on day 6 (3d@6d), and a 6-day exposure group (6d). The addition of deferoxamine decreased cell viability at all three concentrations used ($^*P<0.01$). Moreover, the effect was similar across the three concentrations used. The addition of deferoxamine significantly reduced cell viability in the 72-h (72°) and 6-day (6d) groups, and resulted in a trend of reduced viability in cell groups exposed for 3 days but allowed to recover until day 6 (3d@6d)

viability at 6 days compared to that measured at 72 h. This is typical of established primary neuronal cultures due to their low-mitotic-activity, but normally occurring, cell death. Deferoxamine exposure for 72 h, however, resulted in a significant decrease in cell viability ($P<0.001$). Interestingly, no significant difference in cell viability was found at deferoxamine concentrations of 0.2, 0.4 or 0.8 mg/ml, suggesting that the effect on cell viability at these concentrations was relatively similar. Deferoxamine significantly reduced cell viability after 6 days of exposure relative to controls ($P<0.02$), but was again similar in effect at the three concentrations used. At 6 days, following a 3-day deferoxamine exposure, a similar but non-significant trend to that at 72 h and 6 days was observed, suggesting that a partial recovery may occur following removal of deferoxamine from the medium.

Figure 2 shows the viability of cultures with identical deferoxamine exposure, and the addition of 500 μg/dl $FeCl_3$. In the presence of subtoxic iron concentrations, deferoxamine-induced loss of viability did not occur. No significant difference in MTT absorbance was observed between control (containing sublethal $FeCl_3$ alone) and $FeCl_3$/deferoxamine-exposed cultures.

Hepatoma culture

Hepatoma cell viability after deferoxamine exposure is shown in Figure 3. In the absence of deferoxamine exposure (0 Deferox), increased baseline hepatoma cell viability over time was observed by increased MTT absorbance measured at 6 days compared to that measured at 72 h. This was expected in immortal hepatoma cultures where

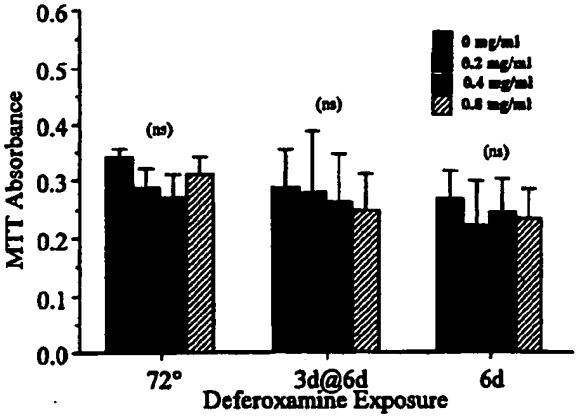


Figure 2 Primary cortical brain cultures exposed to deferoxamine and $FeCl_3$. These cultures were identical to those of Figure 1 with the exception that 500 μg/dl of $FeCl_3$ was added. The presence of iron and deferoxamine together prevented the toxic effects of deferoxamine. No significant differences (ns) were found in the 72-h cultures relative to either the 3- or 6-day cultures

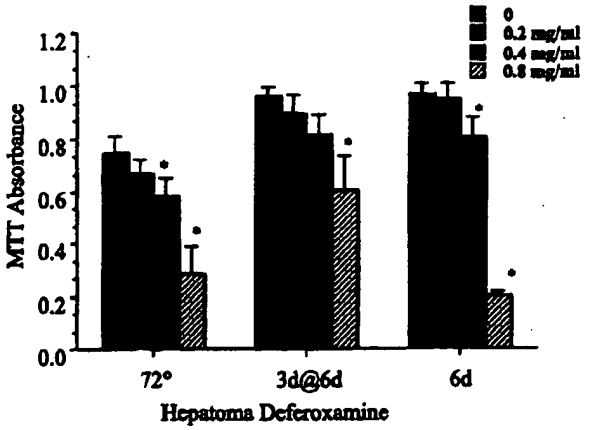


Figure 3 Deferoxamine toxicity in hepatoma cell cultures. Four concentrations of deferoxamine were used under similar conditions as Figure 1. Note that deferoxamine reduced cell viability as determined from MTT absorbance in each of the three conditions and at each of the three concentrations used. In contrast to primary cortical brain cultures, a clear dose-dependent effect was observed ($^*P<0.01$)

continuous cell division typically occurs. Deferoxamine exposure for 72 h or 6 days resulted in significant ($P<0.02$) dose-dependent loss of cell viability as indicated by reduced MTT absorbance. Comparison of cultures exposed to deferoxamine for 3 days and examined at day 6, unlike cultures exposed for 6 days, did not reveal a significant difference in hepatoma cell loss.

An effect similar to that observed in primary cortical brain cultures was also found in combined $FeCl_3$/deferoxamine hepatoma cultures (Figure 4). In the presence of $FeCl_3$, deferoxamine-induced cell loss did not occur, although the normal increase in cell numbers that occurs in hepatoma cell cultures over time was not seen (compare Figures 3 and 4). This indicates that although the addition of $FeCl_3$ blocks the deferoxamine-induced cell loss, the presence of

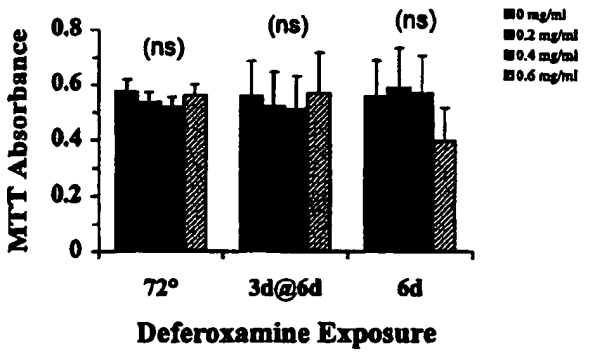


Figure 4 Hepatoma cultures with iron. These cultures were identical to those of Figure 3 with the exception that 500 μg/dl of $FeCl_3$ was added. The presence of iron and deferoxamine together prevented the toxic effects of deferoxamine. No significant difference was found between the groups (ns)

viability at 6 days compared to that measured at 72 h. This is typical of established primary neuronal cultures due to their low-mitotic-activity, but normally occurring, cell death. Deferoxamine exposure for 72 h, however, resulted in a significant decrease in cell viability ($P<0.001$). Interestingly, no significant difference in cell viability was found at deferoxamine concentrations of 0.2, 0.4 or 0.8 mg/ml, suggesting that the effect on cell viability at these concentrations was relatively similar. Deferoxamine significantly reduced cell viability after 6 days of exposure relative to controls ($P<0.02$), but was again similar in effect at the three concentrations used. At 6 days, following a 3-day deferoxamine exposure, a similar but non-significant trend to that at 72 h and 6 days was observed, suggesting that a partial recovery may occur following removal of deferoxamine from the medium.

Figure 2 shows the viability of cultures with identical deferoxamine exposure, and the addition of 500 μg/dl $FeCl_3$. In the presence of subtoxic iron concentrations, deferoxamine-induced loss of viability did not occur. No significant difference in MTT absorbance was observed between control (containing sublethal $FeCl_3$ alone) and $FeCl_3$/deferoxamine-exposed cultures.

Hepatoma culture

Hepatoma cell viability after deferoxamine exposure is shown in Figure 3. In the absence of deferoxamine exposure (0 Deferox), increased baseline hepatoma cell viability over time was observed by increased MTT absorbance measured at 6 days compared to that measured at 72 h. This was expected in immortal hepatoma cultures where

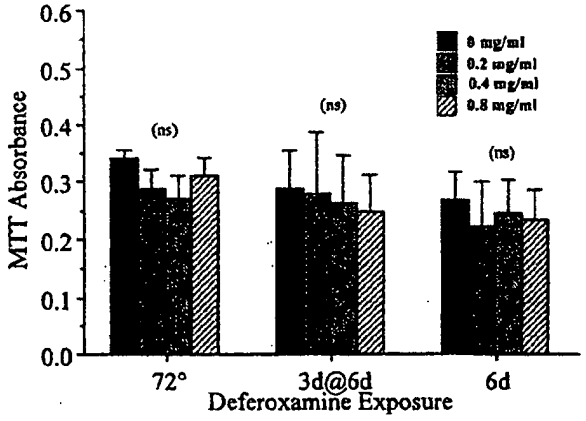


Figure 2 Primary cortical brain cultures exposed to deferoxamine and $FeCl_3$. These cultures were identical to those of Figure 1 with the exception that 500 μg/dl of $FeCl_3$ was added. The presence of iron and deferoxamine together prevented the toxic effects of deferoxamine. No significant differences (ns) were found in the 72-h cultures relative to either the 3- or 6-day cultures

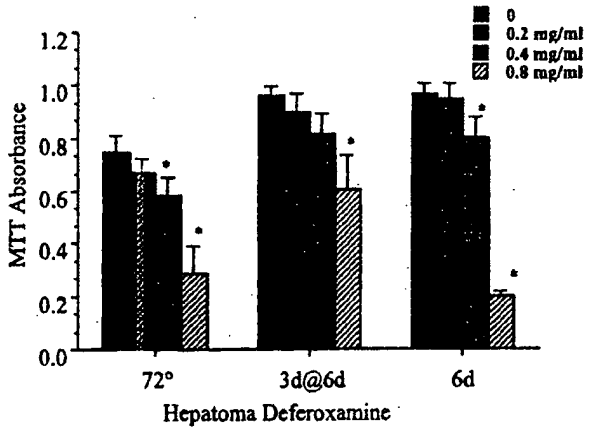


Figure 3 Deferoxamine toxicity in hepatoma cell cultures. Four concentrations of deferoxamine were used under similar conditions as Figure 1. Note that deferoxamine reduced cell viability as determined from MTT absorbance in each of the three conditions and at each of the three concentrations used. In contrast to primary cortical brain cultures, a clear dose-dependent effect was observed (*$P<0.01$)

continuous cell division typically occurs. Deferoxamine exposure for 72 h or 6 days resulted in significant ($P<0.02$) dose-dependent loss of cell viability as indicated by reduced MTT absorbance. Comparison of cultures exposed to deferoxamine for 3 days and examined at day 6, unlike cultures exposed for 6 days, did not reveal a significant difference in hepatoma cell loss.

An effect similar to that observed in primary cortical brain cultures was also found in combined $FeCl_3$/deferoxamine hepatoma cultures (Figure 4). In the presence of $FeCl_3$, deferoxamine-induced cell loss did not occur, although the normal increase in cell numbers that occurs in hepatoma cell cultures over time was not seen (compare Figures 3 and 4). This indicates that although the addition of $FeCl_3$ blocks the deferoxamine-induced cell loss, the presence of

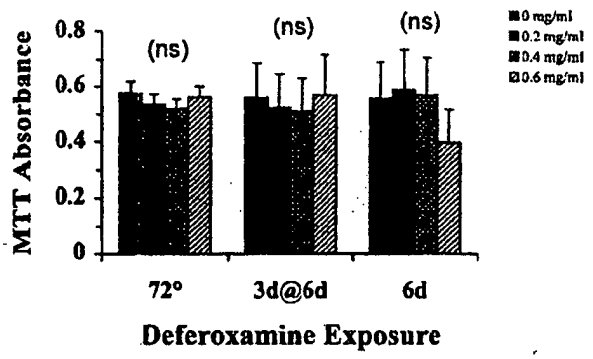


Figure 4 Hepatoma cultures with iron. These cultures were identical to those of Figure 3 with the exception that 500 μg/dl of $FeCl_3$ was added. The presence of iron and deferoxamine together prevented the toxic effects of deferoxamine. No significant difference was found between the groups (ns)

both agents nonetheless affects the increase in cell numbers that occurs in their absence.

One unexpected finding was the protective benefit of the addition of bovine albumin to cultures containing iron. Figure 5 illustrates our findings on the addition of 5% bovine serum albumin to cultures containing either 0, 1000 or 2000 μg/dl of $FeCl_3$ and measured for MTT absorbency 3 h later. The addition of 5% albumin significantly reduced iron-induced toxicity in each of the experimental conditions relative to matched controls.

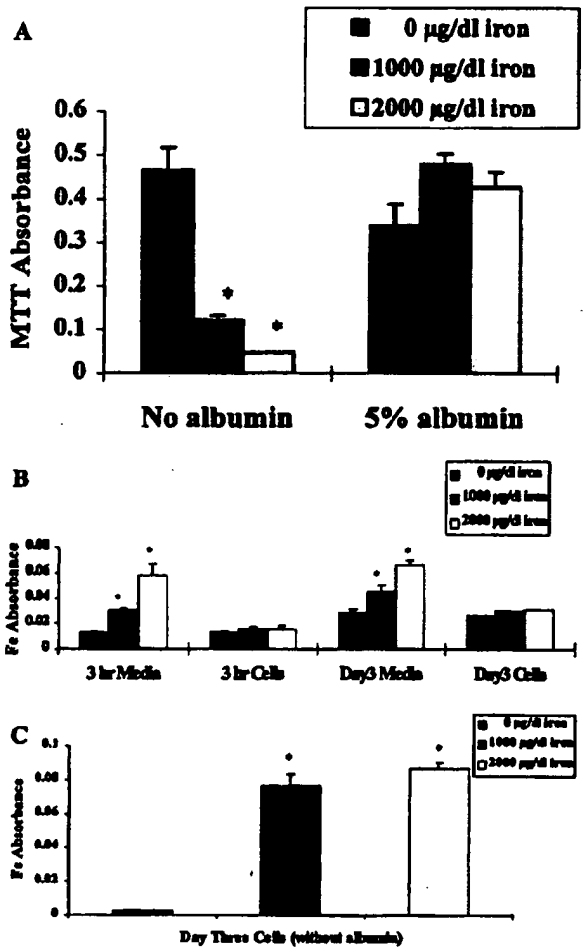


Figure 5 Hepatoma cultures containing 5% bovine albumin and $FeCl_3$. Cultures were as described previously with the exception that both albumin (5%) and $FeCl_3$ were added. (A) Cultures containing $FeCl_3$ at concentrations of 0, 1000 and 2000 μg/dl and measured for MTT absorbance 3 h after exposure to the iron. The presence of albumin significantly reduced ($^*P<0.0001$) the toxicity of the iron at these two levels. (B) Measurement of the amount of iron (as denoted by absorbance) in either the medium or cells exposed to iron and albumin. The medium and cells were measured at 3 h or 3 days after iron exposure. Note that in all cultures, the amount of iron in the medium was significantly higher than in the cells. More importantly, the amount of iron in the control cells was similar to the experimental conditions, suggesting that bovine albumin effectively bound the iron and prevented its entry into the cells ($^*P<0.0001$). (C) Measurement of the amount of iron in cells not exposed to albumin. The three iron concentrations were the same as in (A) and (B). Note that in the absence of albumin, the cells contained significantly greater amounts of iron ($^*P<0.001$)

The addition of serum albumin is a widely accepted method for improving cell growth in cultures. Our observation of increased viability of cells exposed to both iron and albumin was potentially explained by the general benefit of its addition to the cultures. Alternatively, albumin may act to bind iron in the medium, thereby reducing its access to cells. To explore this point, we measured the amount of iron in the medium and within cells of these cultures. For this, the medium was removed from the cultures and measured for iron. The cells were washed three times in iron-free medium, lysed and collected for similar measurement. Figure 5B depicts our observations of the amount of iron in the medium and cells following 3 h or 3 days of exposure. The presence of albumin significantly reduced the amount of iron in cells with values similar to controls. In contrast, the medium from the experimental groups had significantly greater quantities of iron and suggested that one of the benefits of the albumin was to limit the amount of iron that entered the cells. Collectively, these two series of experiments (Figure 5A and B) suggest that serum albumin may have protective benefits comparable to deferoxamine without its cellular toxicity.

Discussion

After ingestion, iron is absorbed as the ferrous form in the duodenum and jejunum and then converted to the ferric form by gastrointestinal mucosal cells. It is transported to body tissues by a β1-globulin in blood. Within the cell, iron is transported to the mitochondria where it becomes localized in the mitochondrial cristae. Iron toxicity is thought to result from interruption of intermediary metabolism and failure of ATP generation in mitochondria, possibly due to iron acting as an electron sink, which shunts electrons away from the cytochrome system. Because detoxification is often ineffective and no therapies are available to interrupt the cellular mechanism of toxicity, outcome is often dependent on the dose ingested and the time elapsed before medical care is sought (for review, see Ref.[11]).

Current therapy is designed to prevent absorption and then to remove absorbed iron (chelation). Cellular iron uptake occurs rapidly, such that an absorbed iron dose is generally cleared from the serum within 6–8 h after acute intoxication. Typically, a toxic ingestion has already been absorbed before patients seek medical care. Iron toxicity increases proportionally to serum levels such that

both agents nonetheless affects the increase in cell numbers that occurs in their absence.

One unexpected finding was the protective benefit of the addition of bovine albumin to cultures containing iron. Figure 5 illustrates our findings on the addition of 5% bovine serum albumin to cultures containing either 0, 1000 or 2000 μg/dl of $FeCl_3$ and measured for MTT absorbency 3 h later. The addition of 5% albumin significantly reduced iron-induced toxicity in each of the experimental conditions relative to matched controls.

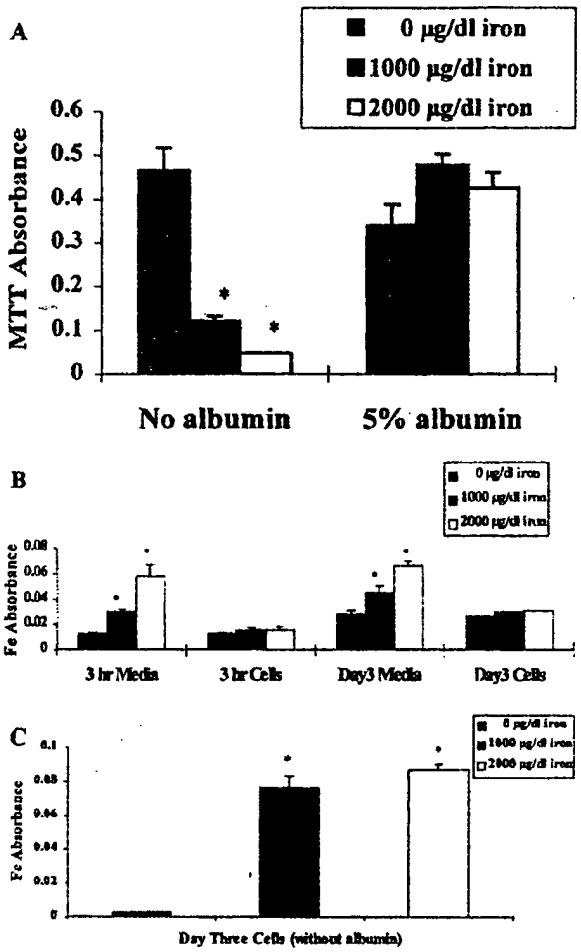


Figure 5 Hepatoma cultures containing 5% bovine albumin and $FeCl_3$. Cultures were as described previously with the exception that both albumin (5%) and $FeCl_3$ were added. (A) Cultures containing $FeCl_3$ at concentrations of 0, 1000 and 2000 μg/dl and measured for MTT absorbance 3 h after exposure to the iron. The presence of albumin significantly reduced ($^*P<0.0001$) the toxicity of the iron at these two levels. (B) Measurement of the amount of iron (as denoted by absorbance) in either the medium or cells exposed to iron and albumin. The medium and cells were measured at 3 h or 3 days after iron exposure. Note that in all cultures, the amount of iron in the medium was significantly higher than in the cells. More importantly, the amount of iron in the control cells was similar to the experimental conditions, suggesting that bovine albumin effectively bound the iron and prevented its entry into the cells ($^*P<0.0001$). (C) Measurement of the amount of iron in cells not exposed to albumin. The three iron concentrations were the same as in (A) and (B). Note that in the absence of albumin, the cells contained significantly greater amounts of iron ($^*P<0.001$)

The addition of serum albumin is a widely accepted method for improving cell growth in cultures. Our observation of increased viability of cells exposed to both iron and albumin was potentially explained by the general benefit of its addition to the cultures. Alternatively, albumin may act to bind iron in the medium, thereby reducing its access to cells. To explore this point, we measured the amount of iron in the medium and within cells of these cultures. For this, the medium was removed from the cultures and measured for iron. The cells were washed three times in iron-free medium, lysed and collected for similar measurement. Figure 5B depicts our observations of the amount of iron in the medium and cells following 3 h or 3 days of exposure. The presence of albumin significantly reduced the amount of iron in cells with values similar to controls. In contrast, the medium from the experimental groups had significantly greater quantities of iron and suggested that one of the benefits of the albumin was to limit the amount of iron that entered the cells. Collectively, these two series of experiments (Figure 5A and B) suggest that serum albumin may have protective benefits comparable to deferoxamine without its cellular toxicity.

Discussion

After ingestion, iron is absorbed as the ferrous form in the duodenum and jejunum and then converted to the ferric form by gastrointestinal mucosal cells. It is transported to body tissues by a β1-globulin in blood. Within the cell, iron is transported to the mitochondria where it becomes localized in the mitochondrial cristae. Iron toxicity is thought to result from interruption of intermediary metabolism and failure of ATP generation in mitochondria, possibly due to iron acting as an electron sink, which shunts electrons away from the cytochrome system. Because detoxification is often ineffective and no therapies are available to interrupt the cellular mechanism of toxicity, outcome is often dependent on the dose ingested and the time elapsed before medical care is sought (for review, see Ref.[11]).

Current therapy is designed to prevent absorption and then to remove absorbed iron (chelation). Cellular iron uptake occurs rapidly, such that an absorbed iron dose is generally cleared from the serum within 6–8 h after acute intoxication. Typically, a toxic ingestion has already been absorbed before patients seek medical care. Iron toxicity increases proportionally to serum levels such that

moderate toxicity is likely with levels >500 mg/dl, and levels >1000 mg/dl are potentially lethal.[2,12] Since decontamination techniques are frequently inadequate or too late to prevent absorption of toxic doses, clinical intervention typically attempts to remove absorbed iron by chelation. Despite lack of proven efficacy, deferoxamine administration is currently the standard of treatment for acute iron intoxication.[2]

Although deferoxamine effectively chelates iron, the recommended maximum dose of deferoxamine is frequently unable to prevent iron toxicity. Deferoxamine chelates iron from ferritin and hemosiderin, forming stable complexes, which prevent the chelated iron from participating in chemical reactions. The iron–deferoxamine complex (ferrioxamine) is excreted primarily by the kidney. The efficacy of chelation therapy is limited by deferoxamine's iron-binding capacity (1.0 mg deferoxamine binds up to 0.085 mg iron) and deferoxamine toxicity. Ingestion of >60 mg/kg of elemental iron is potentially lethal in children, with many children in our experience presenting after ingestion of 100–300 mg/kg. Because iron is generally cleared rapidly from the serum after ingestion, effective therapy for a 60 mg/kg ingestion should theoretically chelate at least 10 mg iron/kg/h (although the actual time interval during which ingested iron must be removed to prevent toxicity is unknown). The current maximum recommended deferoxamine infusion rate of 15 mg/kg/h would be expected to chelate only 1.28 mg/kg/h. Hence, chelation therapy at maximum recommended doses cannot be expected to chelate a significant portion of a potentially toxic dose of ingested iron. Accordingly, despite recommendations that deferoxamine be infused at rates not exceeding 15 mg/kg/h (package insert), higher doses have been used in an attempt to remove absorbed iron. Continuous deferoxamine infusion rates up to 35 mg/kg/h have been used for treatment of iron overdose with apparent success.[13] Successful use of high-dose deferoxamine treatment of iron overload in thalassemic patients has been reported.[5,14,15] High-dose intravenous therapy has been recommended to achieve rapid removal of excess iron.[15] However, enthusiasm for high-dose infusion has been tempered by reports of deferoxamine toxicity. High rates of deferoxamine infusion produce hypotension, which can be effectively treated by aggressive fluid resuscitation (unpublished personal observation). Several authors have noted that high-dose deferoxamine can cause severe toxicity, as can normal doses in patients with a low iron burden[5]. Prolonged infusion or high infusion rates are also associated with ototoxicity,[16] ocular toxicity,[17] renal failure,[18–20] ARDS,[8] and myocardial depression and hypotension.[7] These toxic effects may be additive and difficult to distinguish from similar effects of iron poisoning. Whether deferoxamine toxicity results primarily from hemodynamic changes induced by high-dose deferoxamine or from direct cellular toxicity has not been well documented.

Our model of deferoxamine cytotoxicity in cell cultures eliminates many compounding problems associated with whole-body or whole-animal administration (e.g., pulmonary or circulatory effects). Hepatoma cultures were used because of the prominence of hepatic injury in iron poisoning. Because tumor cell lines tend to be more vigorous than primary cultures, simultaneous evaluation in a primary cell culture was also performed using fetal rat brain cultures. In contrast to hepatoma cells, established primary cortical brain cell lines are fairly static and are not mitotically active. Primary cortical brain cultures are generally unable to compensate for cell loss by continued cell division as that which occurs in hepatoma cultures. Our findings demonstrate a cytotoxic effect of deferoxamine in cell culture documenting direct cytotoxicity in the absence of circulatory factors (Figures 2–4). Interestingly, major differences between the two cell lines were observed. Primary cortical brain cells like hepatoma cells evidenced reduced cell viability when exposed to deferoxamine. However, unlike hepatoma cells, cortical brain cells were similarly affected by deferoxamine at concentrations ranging from 0.2 to 0.8 mg/ml. However, when the cultures were examined at earlier time points, e.g., 2–3 days, a dose–response relationship was observed (data not shown). Thus, the fourfold increase in deferoxamine level, which had only limited dose-related effect on cell viability at day 6, presumably reflects the convergence of earlier cell loss. In contrast, hepatoma cells evidenced a dose-dependent reduction in cell viability at day 6, although determination of effects at low deferoxamine concentrations was problematic and partially masked by continued cell proliferation.

Several studies suggest that deferoxamine toxicity can be reduced by avoiding excessive chelation, particularly in the presence of low iron stores.[5,21] In support of this, ototoxicity in thalassemic patients was more likely when the deferoxamine/serum ferritin ratio exceeded 0.027.[16] While evaluating the antiparasitic effect of deferoxamine in cell culture, Glickstein *et al*[22] noted a deferoxamine-induced reduction of mammalian cell proliferation that appeared to result from deferoxamine's capacity for scavenging intracellular iron. These reports suggest that deferoxamine toxicity is associated with excessive iron chelation. Moreover, they are consistent with our findings that

deferoxamine alone induces cell death in culture, but that the addition of $FeCl_3$ at subtoxic levels prevents deferoxamine-induced cellular toxicity (Figures 2 and 4).

Similar to our observations, Lee and Wurster[23] found that addition of deferoxamine to cultures of tumor cell lines (neuroblastoma and glial) significantly reduced cell viability. The addition of stoichiometric amounts of ferric ions, however, did not decrease the cytotoxic effect of deferoxamine. However, iron-induced cytotoxicity may occur at the doses they employed, which exceeded those used in our study. We have observed that high-dose deferoxamine exposure results in cell death in both primary cortical brain and hepatoma cell cultures, which is reduced by concurrent subtoxic iron exposure.

One surprising finding was the beneficial effect of the addition of bovine albumin to our cell cultures. It is common practice to add albumin to cell cultures to increase cell viability. The beneficial effect of albumin added to cultures containing iron were, in this respect, not surprising. However, further investigation revealed that the iron in the medium failed to enter the cells and suggested that serum albumin may non-specifically bind iron and thereby prevent it from entering the cells (Figure 5A and B). Potentially, albumin may be as effective in removing iron from serum and extracellular fluids as deferoxamine or recent alternatives,[24,25] without their side effects. We are currently evaluating the beneficial effects of administration of serum albumin in rats that have received injections of iron.

In summary, we have observed that high-dose deferoxamine exposure results in cell death in both primary cortical brain and hepatoma cell cultures. Moreover, this cell death is reduced by concurrent subtoxic iron exposure. Because prolonged exposure to high-dose deferoxamine has direct cellular toxicity, its use in humans cannot be safely recommended without further study. Our findings are consistent with clinical observations that deferoxamine toxicity is more common in patients without excessive iron overload, or when use is extended beyond the period when iron overload would be expected. These results support the hypothesis that deferoxamine toxicity is associated with excessive iron chelation. Improved understanding of the limitations and potential toxicity of current therapy and of the mechanisms of iron toxicity may enable improvements that reduce mortality of acute iron intoxication.

Acknowledgements

This work was funded by grants from the John Mace Pediatric Fund (D.W.C. and M.A.K.), the Michael and Helen Schaffer Foundation (M.A.K.), and Department of the Army (DAMD17-97-2-7016 to M.A.K.), but does not reflect the position of the government or NMTB.

References

1 Whitten CF, Brough AJ. The pathophysiology of acute iron poisoning. *Clin Toxicol* 1971; 4: 585–595.
2 Berkowitz ID, Banner W, Rogers MC. Poisoning and the critically ill child. In: Rogers MC, (ed.), *Textbook of Pediatric Intensive Care*, 1992. Williams and Wilkins, Baltimore, MD.
3 Leikin S, Vossough P, Mochiv-Fatemi F. Chelation therapy in acute iron poisoning. *J Pediatr* 1969; 71: 425–430.
4 Fontecave M, Pierre JL. Iron: metabolism, toxicity and therapy. *Biochimie* 1993; 75: 767–773.
5 Giardina PJ, Grady RW. Chelation therapy in beta-thalassemia: the benefits and limitations of desferrioxamine. *Semin Hematol* 1995; 32: 304–312.
6 Adamson IY, Sienko A, Tenenbein M. Pulmonary toxicity of deferoxamine in iron-poisoned mice. *Toxicol Appl Pharmacol* 1993; 120: 13–19.
7 Bentur Y, McGuigan M, Koren G. Deferoxamine (desferrioxamine). New toxicities for an old drug. *Drug Saf* 1991; 6: 37–46.
8 Tenenbein M, Kowalski S, Sienko A, Bowden DH, Adamson IY. Pulmonary toxic effects of continuous desferrioxamine administration in acute iron poisoning. *Lancet* 1992; 339: 699–701.
9 Howland MA. Risks of parenteral deferoxamine for acute iron poisoning. *J Toxicol Clin Toxicol* 1996; 34: 491–497.
10 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55–63.
11 de Montalembert M. Iron chelation in 1998. *Transfus Clin Biol* 1998; 5: 353–356.
12 Henretig FM, Temple AR. Acute iron poisoning in children. *Clin Lab Med* 1984; 4: 575–586.
13 Shannon M. Desferrioxamine in acute iron poisoning. *Lancet* 1992; 339: 1601.
14 Freedman MH, Grisaru D, Olivieri N, MacLusky I, Thorner PS. Pulmonary syndrome in patients with thalassemia major receiving intravenous deferoxamine infusions. *Am J Dis Child* 1990; 144: 565–569.
15 Cohen AR, Mizanin J, Schwartz E. Rapid removal of excessive iron with daily, high-dose intravenous chelation therapy. *J Pediatr* 1989; 115: 151–155.

16 Sacco M, Meleleo D, Tricarico N, Greco Miani A, Serra E, Parlatore L. Evaluation of desferrioxamine ototoxicity in thalassemic patients. Follow-up over a 5-year period and results. *Minerva Pediatr* 1994; 46: 225–230.
17 Mehta AM, Engstrom RE Jr, Kreiger AE. Deferoxamine-associated retinopathy after subcutaneous injection. *Am J Ophthalmol* 1994; 118: 260–262.
18 Koren G, Bentur Y, Strong D, Harvey E, Klein J, Baumal R, Spielberg SP, Freedman MH. Acute changes in renal function associated with deferoxamine therapy. *Am J Dis Child* 1989; 143: 1077–1080.
19 Cianciulli P, Sorrentino F, Forte L, Palombi M, Papa G, Meloni C, Taccone Gallucci M, Casciani CU. Acute renal failure occurring during intravenous desferrioxamine therapy: recovery after haemodialysis. *Haematologica* 1992; 77: 514–515.
20 Cianciulli P, Sollecito D, Sorrentino F, Forte L, Gilardi E, Massa A, Papa G, Carta S. Early detection of nephrotoxic effects in thalassemic patients receiving desferrioxamine therapy. *Kidney Int* 1994; 46: 467–470.
21 Gabutti V, Piga A. Results of long-term iron-chelating therapy. *Acta Haematol* 1996; 95: 26–36.
22 Glickstein H, Breuer W, Loyevsky M, Konijn AM, Shanzer A, Cabantchik ZI. Differential cytotoxicity of iron chelators on malaria-infected cells *versus* mammalian cells. *Blood* 1996; 87: 4871–4878.
23 Lee YS, Wurster RD. Deferoxamine-induced cytotoxicity in human neuronal cell lines: protection by free radical scavengers. *Toxicol Lett* 1995; 78: 67–71.
24 Dragsten PR, Hallaway PE, Hanson GJ, Berger AE, Bernard B, Hedlund BE. First human studies with a high-molecular-weight iron chelator. *J Lab Clin Med* 2000; 135: 57–65.
25 Bergamini S, Rota C, Staffieri M, Tomasi A, Iannone A. Prooxidant activity of ferrioxamine in isolated rat hepatocytes and linoleic acid micelles. *Chem Res Toxicol* 1999; 12: 365–370.